Abstract of Dissertation Presented to the Graduate Council
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

STUDIES ON AGENTS WHICH MODIFY MAST CELL STIMULATION-SECRETION COUPLING

By

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This dissertation is dedicated to Tom who is both my best friend and my husband. Together with him I celebrate personal growth and development.

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PREFACE

"Of all intellectual activity, science alone has flourished in the last centuries, science alone has turned out to have the kind of universality among men which the times require."

J. Robert Oppenheimer

This dissertation is composed of an introductory chapter, two chapters written in standard manuscript style and a final conclusion and significance chapter. Due to this format, some material may appear redundant. Please accept any repeated material as important to the overall contribution of this research to further understandings of mast cell stimulation-secretion coupling.

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Mast cells are long-lived cells which reside in connective tissue and tissues surrounding blood vessels, meninges, sheaths of peripheral nerves and submucosa of the small intestine and respiratory system. They respond to a variety of peptides and participate in concert with bound IgE and antigen to release mediators of immediate hypersensitivity.

The present, detailed investigations were conducted with purified, normal mast cells and the results presented in terms of 1) selective effects of anti-inflammatory steroids and 2) characterization of actions of tumor-promoting phorbol esters upon mast cells stimulated with IgE-dependent secretagogues, peptide-basic agents or the calcium ionophore A23187.

With respect to the selective effect of anti-inflammatory steroids, the interesting findings were 1) long-term (18-21 hr) pretreatment

was required for manifestation of inhibitory effects, 2) the inhibitory effect was limited to anti-inflammatory types of steroids, 3) a specific glucocorticoid receptor mechanism was involved, 4) inhibition of release of histamine and arachidonic acid resulted when cells were stimulated with IgE-dependent secretagogues, 5) no inhibition was noted when cells were stimulated with polypeptide-basic agents or ionophore A23187, and 6) IgE-dependent secretagogue stimulated calcium flux was decreased.

Characterization of the effects of phorbol esters on rat mast cell secretion revealed that 1) phorbol esters tremendously potentiated ionophore A23187 stimulated release of histamine, 2) extracellular calcium was required, 3) histamine release by IgE-like secretagogues was potentiated by the presence of the phospholipid phosphatidylserine and phorbol ester, 4) structure-activity relationships suggested a single mechanism of action, perhaps mediated by a single receptor, 5) phorbol esters either alone or with ionophore A23187 increased phosphorylation of mast cell proteins, and 6) mast cells have low levels of calcium-phospholipid-dependent protein kinase to which phorbol esters bind.

CHAPTER ONE
INTRODUCTION

General Characterization of Mast Cells and Histamine Release

Within the Gell and Coombs (1968) scheme for classifying immunological responses, histamine release from mast cells (and basophils) comprises the type I sensitivity commonly called immediate, anaphylactic or reagin-dependent hypersensitivity. This reaction occurs upon re-exposure to a specific antigen which interacts with its immunoglobulin E (IgE) antibody bound to the surface of mast cells and basophils and triggers a sequence of activation-secretion coupling events which culminate in the release of several inflammatory mediators (Altman, 1981). Minimum requirements for an immediate hypersensitive event are allergen and mast cell or basophil with specific IgE bound to the cell surface Fc receptor. Initial exposure to an allergen requires collaboration between it, macrophages and T lymphocytes which stimulate B lymphocytes to differentiate to plasma cells and synthesize and release IgE-class antibodies. Investigations into the regulation of the IgE antibody formation response have led to the hypothesis that certain IgE-class specific subpopulations of both helper and suppressive T cells work together with B cells to modulate the antibody response (Tadamitsu, 1982). Normally, suppressive mechanisms predominate to keep IgE levels low; therefore; allergic diseases may represent a "breakthrough" phenomenon (Katz, 1978a, 1978b).

Mast cells to which IgE binds, reside predominantly in connective tissue and skin surrounding blood vessels, meninges, sheaths of peripheral nerves and submucosa of the small intestine and respiratory system. These are long-lived cells, capable of regranulation in contrast to their circulating counterparts, the basophils, which are short-lived, incapable of regranulation and can move into tissues in response to chemo-attractants (Altman, 1981). Both cell types store certain mediators of hypersensitivity reactions, such as histamine which is bound to a water-insoluble complex of protein and heparin within cytoplasmic granules. Rat peritoneal mast cells reportedly contain 250-500 granules per cell, and this may comprise up to 70% of the cellular dry weight (Bergendorff and Uvnas, 1973).

Release of histamine and other mediators in an immediate hypersensitivity episode is the outcome of a complex biochemical series of events beginning with activation of the cell surface IgE receptor. Several investigative groups have reported that normal rat mast cells have approximately 2×10^5 IgE receptors/cell (Conrad et al., 1975; Ishizaka et al., 1975; Mendoza and Metzger, 1976). Cross-linking of receptor-bound IgE by multi-valent antigen, anti-IgE or direct cross-linking of IgE receptors by anti-receptor IgE triggers the sequence of activation-secretion events (Ishizaka and Ishizaka, 1968; Ishizaka et al., 1971; Ishizaka and Ishizaka, 1978). Activation of a serine esterase closely associated with IgE receptors has been postulated as a very early event in stimulus activated mast cells and may play a general role in secretory cell activation (Taylor and Sheldon, 1974). According to the mobile receptor hypothesis of

Cuatrecasas (1974), membrane-associated receptors and enzymes acquire affinities to form complexes upon receptor occupation and may thus "uncover" active sites of these enzymes of mediator release. Other enzymes which fit such a hypothesis include phospholipase A_2, phospholipase C, protein kinases such as the calcium and phospholipid dependent protein kinase (Ca/PL-PK) and the phospholipid methyltransferases. While different classes of secretagogues may utilize different enzymes in the initial transmembrane signalling sequences, it is thought they employ a common sequence during the secretion phase. This latter phase involves microtubule assembly (Lagunoff and Chi, 1976) which facilitates intracytoplasmic granule movement and culminates in very poorly understood granule-plasma membrane fusion and granule content extrusion.

Degranulation of mast cells and basophils is sensitive to both pH, with optimum release at pH 7, and temperature, secretion is inhibited at 0° or 45°C (Moran et al., 1962; Baxter and Adamik, 1975). There is an absolute requirement for calcium, and in the case of IgE-mediated degranulation, there must be extracellular calcium ions available (Foreman and Monger, 1972; Baxter and Adamik, 1978). Availability of metabolic energy in the form of ATP is a basic prerequisite for histamine release and can be derived either from oxidative phosphorylation or glycolysis. Reports indicate that cellular ATP levels decrease by 30-35% during the release process (Kazimierczak and Diamant, 1978).

From this general description of the mast cell and the complex sequence of events involved in degranulation, we can see that it

entails an active secretory process and has been considered a prototypic secretory cell (Douglas, 1968) having the following distinct components: 1) activation by specific stimuli, 2) extrusion of granular material, and 3) cellular recovery and restoration of granule contents (Kirshner and Viveros, 1972).

Classes of Agents which Release Histamine from Mast Cells

Immediate type hypersensitivity reactions by definition involve antigen and antibody; however other diverse chemical as well as physical stimuli are capable of inducing granule extrusion (Foreman, 1981; Lagunoff et al., 1983; Kazimierczak and Diamant, 1978; Ho et al., 1979). Rather than bridge receptor-bound IgE molecules, these other chemical stimuli interact with specific mast cell receptors to initiate stimulation and secretion. Types of agents capable of eliciting non-cytotoxic mediator release from mast cells and basophils are quite diverse. An abbreviated categorization of these agents central to these investigations includes immunologic agents, lectins, polycationic amines and polypeptides and ionophores.

Within the immunologic agent category fall IgE antibody, IgE generated toward another species IgE (i.e. anti-IgE), anti-receptor IgE and the anaphylatoxins C3a and C5a. Release elicited by the first three agents proceeds via activation of the IgE Fc cell surface receptor, as previously described. Anaphylatoxins C3a and C5a, generated from complement components, are believed to induce degranulation by occupation of their own receptors, independent of the IgE Fc receptor (Siraganian and Hook, 1976; Hartman and Glovsky, 1981), but the requirements for release are identical to the IgE-dependent

mechanism. Both require extracellular calcium, an energy source, are temperature dependent and are potentiated by exogenous phosphatidylserine (PS).

Lectins are hemagglutinins which possess saccharide-specific binding sites and include Jack bean concanavalin A (con A), as well as wheat germ, castor bean and lentil agglutinins. While all these agents reportedly elicit release, most is known about con A induced histamine release. Critical determinants of the extent of con A induced release may vary dependent upon the strain of rat used, presence or absence of extracellular calcium, the degree of cell sensitization and requirement for exogenous PS (Lagunoff et al., 1983). However, there are data which convincingly suggest that con A, tetravalent at physiological pH, cross-links bound IgE by interaction with saccharide moieties located near the Fc region of the IgE molecule (Siraganian and Siraganian, 1975; Fewtrell et al., 1979). Though normal rat mast cells may be quite resistant to con A, cells collected from animals infected with Nippostrongylus brasiliensis, known to increase IgE synthesis, become more sensitive to con A (Keller, 1973). Lastly, monomeric antibody directed against the Fc portion of IgE inhibits con A induced histamine release, indicating that this lectin binds to the Fc region of IgE bound to the mast cell surface (Magro and Bennich, 1977). In Sprague-Dawley rats, the strain used in these investigations, the requirements for con A induced mast cell degranulation are identical to those for IgE-induced release.

The prototypic polycationic amine releasing agent is compd 48/80, a polymerization product of equimolar p-methoxy-N-methyl phenethylamine

and formaldehyde. Polypeptides in this group include somatostatin, substance P, bradykinin, ACTH and neurotensin (see table compiled by Lagunoff et al., 1983). These agents share a common feature, the presence of at least two basic residues; it has been suggested that any molecule possessing two basic groups separated by an aliphatic chain of about five carbons or an aromatic skeleton of corresponding length is likely to be a mast cell histamine liberator (Paton, 1958). Recent evidence suggests that compd 48/80 and somatostatin induce histamine secretion in mast cells by interacting with the same cell surface receptor (Theoharides et al., 1981). Release induced by agents of this class differ from the above classes in that there is no enhancement by exogenous PS, no requirement for extracellular calcium, and no stimulation of phospholipid methylation (Hirata et al., 1979). This suggests that initial events of the activation-secretion response of mast cells may be different for polycationic amines and basic polypeptides than IgE-like secretagogues.

Ionophores, compounds which facilitate transport of ions through a lipid barrier separating two aqueous environments, serve as carriers for various ions. The most completely described is known simply as A23187, a monocarboxylic antibiotic isolated from Streptomyces chartreusensis; it is capable of eliciting secretion from a wide variety of cells, including mast cells. In the secretory process, the ionophore A23187 functions as a calcium carrier, is dependent upon extracellular calcium and is capable of bypassing the regulatory effect of cAMP upon mast cell secretion (Foreman et al., 1976). It is likewise indifferent to the presence of exogenous PS.

For these investigations, we have chosen at least one agent from each of these four groups: sheep anti-rat IgE (anti-IgE); the antigen ovalbumin, con A; compd 48/80 or somatostatin and the calcium ionophore A23187.

Other Mediators Released by Mast Cells

Histamine is but one of many pharmacological principles released by mast cells. All mediators can be divided into two broad but useful categories; those like histamine, which are stored and those which are generated as a consequence of specific activation of the target cell (Kazimierczak and Diamant, 1978). Pharmacological action, physiochemical characteristics and metabolism of both preformed and generated mediators have been reviewed in detail by Ho et al. (1979). Preformed mediators are stored within basophil and mast cell membrane bound granules composed largely of proteoglycan and protein and include histamine, serotonin (in some species, Yurt and Austen, 1977), eosinophil chemotactic factor of anaphylaxis (ECF-A), neutrophil chemotactic factor (NCF) and heparin. Included with stored, granule-associated mediators are enzymes also released following cell activation. Chymase, a chymotrypsin-like enzyme, is presumed to be stored with serotonin at its active site in rat mast cell granules (Lagunoff and Pritzl, 1976). N-Acetyl-β-D-glucosaminidase and arylsulfatase A are two other stored enzymes released during degranulation and are thus presumed to reside in secretory granules (MacDonald-Lynch et al., 1978).

Mediators generated subsequent to mast cell activation consist predominantly of prostaglandins (PGs) and leukotrienes (LTs), the

oxidative products of arachidonic acid (AA). They are generated by the release of AA from phospholipid membrane stores following secretagogue-induced perturbation which is believed to allow expression of phospholipases (Flower and Blackwell, 1976).

Metabolism of AA occurs by two independent pathways: the cyclooxygenase and lipoxygenase pathways. In the rat and human mast cell, the former pathway initially generates the unstable cyclic endoperoxides PGG_2 and PGH_2 which are converted predominantly to PGD_2 by the action of a specific synthetase (Lewis and Austen, 1981). Metabolism *via* the lipoxygenase pathway generates the intermediate 5-hydroperoxyeicosatetraenoic acid (5-HPETE) which is converted to the closely related mono-hydroxyeicosatetraenoic acid (5-HETE) or the unstable intermediate 5S-oxido-trans-7,9-trans,11,14-cis-eicosatetraenoic acid (LTA_4). From LTA_4, two enzymatic pathways have been described, one leading to the potent chemotactic factor LTB_4 (5S,12R,dihydroxy,6,14,cis,8,10-trans-eicosatetraenoic acid) (Borgeat and Samuelsson, 1979) and the other leading to 5S-hydroxy,6R-S-glutathionyl-7,9-trans,11,14-cis-eicosatetraenoic acid (LTC_4) by the addition of glutathione. LTC_4 is then metabolized to the corresponding cysteinylglycine derivative LTD_4 then the cysteine derivative LTE_4 (Lewis *et al.*, 1982). In stimulated human neutrophils, the 5-lipoxygenase pathway is preferentially activated to generate 5-HETE and LTB_4. In contrast, rat and human mast cells elaborate PGD_2 during coupled activation-secretion (Lewis *et al.*, 1982) when stimulated with anti-IgE. LTC_4 has been identified following stimulation of a murine mastocytoma cell line with the calcium ionophore A23187 (Samuelsson *et*

al., 1980). It has been suggested that in human and rat mast cells challenged with IgE-dependent stimuli, only PGD_2 is generated but the secondary cell types recruited may be the source of the other oxidative AA metabolites active in immediate hypersensitivity (Lewis and Austen, 1981).

For the series of experiments described in these chapters, we have chosen to study histamine as the granule-contained secretion marker and AA, in the form of [^{14}C]-AA incorporated in vitro, as the marker generated during mast cell stimulation.

Role of Calcium in Mast Cell Exocytosis

According to Gomperts (1983), only one early event in the mast cell stimulus-response sequence has been unequivocally identified and that is the elevation of cytosolic calcium into the micromolar range. It has been stated that this is the sole necessary precursor of secretion. "Stimulus coupling," first proposed by Douglas and Rubin (1961), has been introduced to emphasize the central role of calcium as a second messenger in secretory processes (Pearce et al., 1983). Experimental results with calcium ionophores such as A23187, ionomycin and chlortetracycline which form lipid-soluble complexes with the cation and directly transfer it across the cell membrane bypassing the normal messenger-receptor interaction, have served as evidence that calcium is the second messenger which couples activation with the granular release (Cochrane and Douglas, 1974; Lichtenstein, 1975; Bennett et al., 1979; Pearce et al., 1983). Though most investigators agree that ionophores transport extracellular calcium to the intracellular spaces,

ionophore-induced histamine release in rat mast cells in the absence of extracellular calcium (thus presumably by mobilization of internal calcium stores) has been reported (Johansen, 1980). A second line of evidence that calcium is the second messenger has centered around mast cell exocytosis following microinjection of calcium ions into the cells while microinjections of magnesium or potassium were without effect (Kanno et al., 1973).

The source of calcium differs dependent upon the type of secretagogue. Antigen-induced, as well as other IgE-dependent exocytosis, requires extracellular calcium, whereas ligands such as compd 48/80, peptide 401 and polylysine induce secretion in the absence of extracellular calcium (Foreman, 1981). Lanthanides, which compete with calcium for extracellular binding sites thought to be the calcium channel, thus prevent calcium transport across membranes and inhibit mast cell histamine secretion induced by IgE-like secretagogues dependent upon extracellular calcium. They do not affect release by compd 48/80, peptide 401 and the like (Foreman and Monger, 1973; Pearce and White, 1981; Amellal and Landry, 1983).

Relationships between histamine secretion and $^{45}Ca^{2+}$ uptake by mast cells have been thoroughly investigated by Foreman et al. (1977). Stimulation with antigen-antibody, dextran or con A induced an uptake of $^{45}Ca^{2+}$ which correlated well with the magnitude of histamine release. PS enhanced both $^{45}Ca^{2+}$ uptake and histamine release. When cells were stimulated with antigen in the presence of non-labeled calcium, then exposed to tracer $^{45}Ca^{2+}$ over a period of 1-5 min, a 64% drop in calcium uptake was noted within 1 min following stimulation

indicating a very transient membrane permeability to the cation. Raised intracellular levels of cAMP produced with theophylline or dibutyryl cAMP inhibited antigen-induced $^{45}Ca^{2+}$ uptake and histamine release, but had no effect when the ionophore A23187 was used as stimulant. It appears, then, that cAMP may inhibit secretion by reducing membrane permeability to calcium and may be involved in limiting calcium entry after cell stimulation.

Evidence for mobilization of membrane or intracellular calcium stores to provide the needed rise of cytosolic calcium ions is given by results of mast cell stimulation in the absence of extracellular calcium following short-term (10 min) pretreatments with chelating agents. Peptide-basic secreting agents including compd 48/80, bradykinin, somatostatin, polylysine and polymyxin B were capable of robust release responses in the absence of calcium (Ennis et al., 1980; Baxter and Adamik, 1978; Cochrane et al., 1982). Based upon such investigation, three pools of calcium have been postulated: 1) calcium very loosely bound to the outer cell membrane which may migrate to the cellular cytosol, 2) a superficial, membrane associated store (removable by brief EGTA exposure) and 3) deeply sequestered calcium stores (removable by prolonged exposure to EGTA (Ennis et al., 1980). While the first store is utilized by both IgE-like and IgE-independent secretagogues as well as ionophores, the second store is mobilized by polypeptide-base type secretagogues. Deeply buried depot translocation may be modulated by occupancy of the two more superficial pools.

Membrane events implicated in mast cell calcium translocation include turnover of phosphatidylinositol (PI) (Gomperts et al., 1980;

Cockcroft and Gomperts, 1979; Kennerly et al., 1979b,c). In rat peritoneal mast cells stimulated with antigen, anti-IgE, con A, chymotrypsin and compd 48/80, turnover of PI and incorporation of radiolabel into PI took place regardless of the presence or omission of extracellular calcium. Concomitant histamine release was either abolished or reduced depending upon calcium requirements of the ligand. This was taken as evidence for involvement of the PI response in the regulation of calcium channels and mobilization of sequestered calcium in the mast cell (Pearce, 1982; Cockcroft and Gomperts, 1979).

Another membrane event implicated in calcium translocation was phospholipid methylation where two membrane-bound enzymes (methytransferases I and II) act sequentially to convert endogenous PE to PC. Methylation has been demonstrated in rat leukemic basophils and peritoneal mast cells during stimulation with antigen, con A and anti-IgE (Crews et al., 1980; Hirata et al., 1979; Ishizaka et al., 1980). Because the methyltransferase inhibitor, 3-deaza-adenosine, blocked phospholipid methylation, calcium influx and histamine release in a dose-dependent manner, it was suggested that phospholipid methylation may be a primary and obligatory event in calcium translocation and histamine release by IgE-dependent mast cell activation.

From available data, it is still not possible to determine the relative contributions of these two pathways of lipid metabolism to the sequence of mast cell activation-secretion coupling events. They may represent events of differing initial activation sequences, events of a complex but unified sequence, or consequential events of cell

activation. Both the PI response and phospholipid methylation will be discussed in more detail in the next section.

The mechanism whereby calcium induces the mast cell exocytotic response is not known. In many systems, the cation produces its effects by interaction with specific binding proteins exemplified by the ubiquitous polypeptide calmodulin, proposed as a universal intracellular receptor for calcium (Means and Dedman, 1980; Cheung, 1980). Calmodulin mediates activity of a number of important enzymes including cyclic nucleotide phosphodiesterase, brain adenylate cyclase, calcium dependent ATPase, phospholipase A, myosin light chain kinase, as well as other specific kinases involved in phosphorylation of membrane and cytosolic proteins (Pearce, 1982).

In smooth muscle cells, neuroleptic drugs reportedly inhibit contraction induced by various stimuli by inhibiting calmodulin (Kerrick et al., 1981). Seven such agents including five phenothiazine derivatives, imipramine and pimozide have recently been studied for their effects upon mast cell secretion evoked by specific antigen, compd 48/80 or ionophore A23187 (Douglas and Nemeth, 1982). An inhibitory potency series generated with the agents for compd 48/80 and A23187 induced release correlated closely with potencies which inhibited phosphodiesterase activation by Ca-calmodulin and also reflected their affinity for binding to calmodulin (Weiss et al., 1980). Specific antigen-evoked secretion was more sensitive to inhibition and gave a different rank order of the neuroleptic agents, an effect shown not to be related to the magnitude of antigen-induced secretion. Since A23187 elicited $^{45}Ca^{2+}$ uptake was not reduced by the

neuroleptics, it was suggested by Douglas and Nemeth that since non-specific membrane effects had been ruled out, their action was distal to the intracellular rise of calcium ions either on the calcium receptor or the processes it activates to effect exocytosis. These results suggested that mast cells may have a calcium-binding protein(s) which resembles calmodulin and that it is involved in receptor-activated exocytosis. Its function may be related to calcium-dependent protein phosphorylation which has been correlated with mast cell degranulation (Sieghart et al., 1978).

Exogenously added calmodulin inhibited mast cell histamine release elicited by compd 48/80, polymyxin B and A23187 (ID_{50} about 2 μM) but not con A (McClain et al., 1983). Calmodulin itself did not elicit release, did not appear to enter the cells and did not compete with compd 48/80 or polymyxin B for cell surface binding sites. EGTA washing of mast cells reduced subsequent calmodulin-induced inhibition. So, release may involve externally-bound calcium but the relationship of this exogenous calmodulin effect to the actual in vivo mechanism remains unknown.

Care must be used in interpreting these results since a very recent report on human platelet work stated that these same agents, namely trifluoperazine, chlorpromazine and W-7 were powerful inhibitors of the calmodulin independent Ca/PL-PK also known as C-kinase (Sanchez et al., 1983). In quin 2 loaded platelets, the secretory response stimulated by phorbol ester, exogenous diacylglycerol (DAG) or collagen was suppressed by the neuroleptics at 20-60 μM at basal levels of cytoplasmic free calcium. Inhibition could be overcome by treatment of

platelets with A23187 (40 nM) which elevated cytoplasmic calcium to 700 nM (sub-threshold for calcium alone to evoke secretion). In contrast, the response to thrombin which was accompanied by elevation in levels of cytosolic free calcium was barely affected. These data suggested that the most prominent effect of phenothiazines, at least on platelets, could be interference with Ca/PL-PK rather than calmodulin-dependent processes.

Though there is sufficient evidence to support the view that elevation of cytosolic calcium is the second messenger in mast cell secretion, subsequent events which calcium initiates or influences remain largely unknown. Likely events, by analogy with other secretory cells or from the results discussed above include involvement of calmodulin, interaction with cyclic nucleotides and cytoskeletal elements such as microfilaments and microtubules, and activation of phospholipases and Ca/PL-PK while methyltransferase activation and PI metabolism may modulate calcium movement.

Phospholipid Metabolism During Mast Cell Activation

Phospholipid methylation. As mentioned in the previous section, the two predominant phospholipid metabolizing systems are the PI cycle and phospholipid methylation. Some evidence for the increase of both these events during mast cell and/or basophil stimulation has been reported (Crews, 1982).

Phosphatidylcholine (PC) is a major constituent of biomembrane phospholipids including those of mast cells and is synthesized by at least two described pathways, the CDP-choline pathway and successive N-methylations of phosphatidylethanolamine (PE) via methyltransferase activity (Kennedy and Weiss, 1956; Hirata et al., 1979).

In normal mast cells, Hirata et al. (1979) have reported a transient rise in membrane phospholipid methylation which precedes histamine release during the early stages of con A-induced activation-secretion coupling, as assessed by the incorporation of [^{3}H]-methyl groups into the mast cell lipid fraction. In the presence of calcium, labeled PC was further metabolized, presumably by phospholipase A_2, to form lysophosphatidylcholine. Both phospholipid methylation and histamine release were inhibited by α-methylmannoside which prevents binding of con A to its cell surface receptors. Similarly, the methyltransferase inhibitors 5'-deoxyisobutylthio-3-deazaadenosine or 3-deazaadenosine (3DZA) plus homocysteine thiolactone inhibited both phospholipid methylation and histamine release. In the absence of calcium, methylation but no subsequent histamine release occurred. When compd 48/80 and ionophore A23187 were employed as degranulating agents, no stimulation of phospholipid methylation was observed. These results were taken to imply that while phospholipid methylation was involved in the activation-secretion signal transduction by con A, it was bypassed by both compd 48/80 and A23187 perhaps indicating different mechanisms of histamine release (Morita and Siraganian, 1981).

Results similar to those described for con A stimulated normal cells were then reported for 2H3 rat basophilic leukemia (RBL) cells sensitized with specific IgE than stimulated with the corresponding antigen and extended to include concomitant inhibition of histamine and [^{14}C]-AA in 3DZA treated cells. More recent studies with RBL cells by Crews et al. (1981) have shown that there was a parallel increase

between [^{3}H]-methyl incorporation into phospholipids and $^{45}Ca^{2+}$ influx which preceded release of histamine and AA release. Thus it was suggested that phospholipid methylation may play a role in calcium flux.

With a slightly different approach, by generating rabbit antibodies to the IgE receptor for RBL cells (anti-RBL) which acted as a divalent anti-receptor binding agent in normal rat mast cells, it was shown that anti-RBL challenge increased phospholipid methylation which reached a maximum at 15 sec then rapidly declined and was followed by an increase of $^{45}Ca^{2+}$ uptake, then histamine release (Ishizaka, 1982). Monomeric antibodies failed to induce any of these changes. With the use of inhibitors described above, corroborative results were reported, again suggesting that phospholipid methylation may be intrinsic for $^{45}Ca^{2+}$ influx and histamine release induced by IgE-receptor stimulation. Additionally, a 3-fold rise of mast cell cAMP, superimposed upon [^{3}H]-methyl incorporation was measured, with a maximum at 15 sec, followed by a sharp decline, then a gradual rise to double basal level again at 3 min following addition of anti-RBL. Methylation inhibitors such as S-isobutyryl-3-deazaadenosine (3-deaza-SIBA) also partially inhibited the initial rise in cAMP suggesting that cAMP may in turn regulate phospholipid methylation. In a dose dependent manner, theophylline, at millimolar levels increased cAMP, inhibited methylation, $^{45}Ca^{2+}$ influx and subsequent histamine release.

More recently, Daeron et al. (1982) have reported that pretreatment of mouse mast cells overnight with 10^{-7} to 10^{-6} M dexamethasone did not itself alter mast cell cAMP levels, but when the cells were stimulated

with specific antigen, phospholipid methylation, $^{45}Ca^{2+}$ influx and histamine release were all inhibited by at least 75%. However, the critical experiments, measurement of cAMP during stimulation were not reported. Thus, while it is still not known how dexamethasone prevents activation of methyltransferases, it does not appear to be by alteration of resting cAMP levels.

Cumulatively, the data can be interpreted to indicate that phospholipid methyltransferases: 1) which effect synthesis of PC to PE are involved in mast cell receptor-mediated activation, 2) selectively increase phospholipid methylation associated with IgE-receptor mediated activation, 3) act prior to an influx of calcium and subsequent histamine release, and 4) when inhibited, likewise inhibit calcium influx as well as histamine and AA release. Therefore, phospholipid methylation may play a critical role in the early events of IgE-mediated mast cell activation-secretion coupling.

PI metabolism. While there is much information on phosphoinositide metabolism and hormone and neurotransmitter action as well as the role of PI hydrolysis as a transducing mechanism in many diverse tissues and cell types (Berridge, 1981; Farese, 1983a; Farese, 1983b), there is very little information on the role of PI in mast cells or basophils. Yet, since the inositol phospholipids have been assigned an important role in mediating actions which generate intracellular calcium signals, as do mast cells, PI involvement in mast cell activation-secretion coupling might be expected.

According to the postulate put forward by Michell and colleagues, PI hydrolysis is coupled to receptor activation and is responsible for

calcium influx or mobilization of intracellular calcium stores. This is supported by observations that the PI cycle (measured directly as hydrolysis or indirectly by increased labeling) occurs with many stimulants that operate *via* receptors and employ calcium as "second messenger" (Farese, 1983a). In this pathway, PI, PtdIns-4,5P_2 and/or PtdIns-4P are hydrolyzed by a phosphodiesterase such as phospholipase C to yield DAG and inositol phosphate(s). DAG can serve as a substrate for diacylglycerol lipase to yield AA, the predominant fatty acid at the 2-position, or diacylglycerol kinase to yield PA which can be picked up by the appropriate CTP transferase and combined with inositol phosphate to reform PI. Relevance of this pathway arises from the following: PA is known to have calcium ionophoric activity, DAG is a known fusogen, and, in concert with PS and calcium, activates Ca/PL-PK while PtdIns-4,5P_2 and PtdIns-4P avidly bind calcium and could modulate membranous calcium stores and/or alter activities of membrane associated enzymes or transport proteins.

Cockcroft and Gomperts (1979) have published evidence for a role of PI turnover in stimulus-secretion coupling in mast cells of rats sensitized to either ovalbumin or the parasitic helminth *Nippostrongylus brasiliensis*. Purified mast cells were preincubated with either [^{3}H]-inositol or ^{32}-P_i for 30 min, and then stimulated with specific antigen, con A or the non-IgE directed secretagogue compd 48/80 for 15 min. Stimulated PI labeling was independent of extracellular calcium though IgE-dependent histamine release was highly calcium dependent. These results were taken as an indication that the mast cell can be placed on the list of tissues and cell types which

exhibit calcium-independent PI responses and thus may represent receptor mediated events proximal to calcium signals. Stimulated PI labeling is believed to be a secondary consequence of the breakdown of PI initiated by either the IgE-directed specific angigen, con A or compd 48/80. At first glance, it seems puzzling that a secretagogue which is independent of extracellular calcium would be involved in the turnover of a phospholipid involved in calcium gating; however compd 48/80 (10 µgm/ml) stimulated PI synthesis was reflected as 2400 dpm ^{32}P-PI/1-2 x 10^5 cells while con A induced PI synthesis was 10-fold greater. Though not stated, these results also suggest that calcium-gating may not be the sole function of PI metabolism in the mast cell.

In a subsequent report (Gomperts et al., 1980), an extensive table of tissues which can be stimulated by the ionophore A23187 and also exhibit a PI response was used as evidence to suggest that the PI response indicates a receptor mediated biochemical step in the activation of calcium channels as postulated by Michell (1975).

Activation of metabolism of the predominant mast cell phospholipids was investigated by incorporation of $^{32}P_i$ into individual classes of phospholipids during stimulation by anti-IgE, con A, compd 48/80 or calcium ionophore A23187 (Kennerly et al., 1979a). Increased incorporation into PA, PI and PC (4- to 10-fold increases) occurred within 15 min while no significant incorporation into PS, PE or sphingomyelin (SM) was discerned. Kinetic analyses of phospholipid metabolism during anti-IgE stimulation showed that PA labeling increased most rapidly from 15 sec to 2 min after stimulation while PI

and PC labeling increased more slowly and reached a maximum after 6 min of stimulation. Major changes in PA labeling occurred before mediator release, PI and PC changes were concomitant with histamine release. These increases of $^{32}P_i$ incorporation were presumed to indicate that mast cell receptor-induced stimulation generated DAG which is then converted to PA, PI and PC. These results support the hypothesis that selective mast cell phospholipid metabolism may play a critical role in the biochemical events which control mediator release.

Since DAG is a precursor for each of the phospholipids, experiments were designed to determine levels of DAG during mediator release from mast cells stimulated with compd 48/80 (Kennerly et al., 1979b). In [^{3}H]-AA prelabeled cells, a small but significant absolute label accumulation in DAG (250-300 cpm/3 x 10^5 cells) was noted within 60 sec of 48/80 addition. Unfortunately, this increase was too small to demonstrate an absolute loss from any of the labeled presursor lipids PI, PC, PE and triglyceride. In broken cell preparations, 2-[1-^{14}C]-arachidonoyl-DAG was rapidly converted to free AA, monoacylglycerol and triglyceride. Substrate preference of 2-[1-^{14}C]-arachidonyl-DAG over 1-[1-^{14}C]-arachidonyl-PC suggested that degradation was mediated by a DAG lipase, demonstrated in mast cells by Lewis et al. (1979). Since the time course of ^{32}P-PA accumulation in the previously described investigations was more rapid than for DAG, it was suggested that mast cell DAG kinase may initially remove newly formed DAG until stimulated DAG production exceeds the enzyme's capacity, then the abrupt rise in DAG levels is measured. Postulated roles for DAG and its metabolites in mast cell secretion included facilitation of membrane fusion and

substrate for AA-derived mediator formation. These same observations were extended to anti-IgE stimulated mast cells (Kennerly et al., 1979c).

Results of experiments conducted with [^{3}H]-glycerol loaded mast cells stimulated with either ionophore A23187 or compd 48/80 and designed to study PI breakdown in activated mast cells have been published by Ishizuka and co-workers (1983). Following stimulation of cells by A23187, radioactivity in both PA and PI dropped slightly at 10 sec then went on to accumulate at 1.5 times the starting level at 60 sec. In contrast, radioactivity in DAG progressively increased to a 3-fold increase by 5 min. To investigate the possibility that A23187-induced activation stimulated de novo PI and PA synthesis, uptake of [^{3}H]-glycerol was investigated during stimulation. Enhanced uptake of [^{3}H]-glycerol into PI and PA by 7- and 4-fold, respectively, was measured. Though the percent total histamine released was comparable, compd 48/80 was much less effective in accelerating PI metabolism than A23187. In another report, Ishizuka and Nozawa (1983) have discussed results of the same experimental design but with specific antigen as the stimulant. In presence of 0.5 mM magnesium, antigen stimulation (Ascaris suum extracts coupled to 2,4-dinitrophenyl) induced a loss of 30% PI from mast cells preloaded with [^{3}H]-glycerol followed by a PI resynthesis to 1.5-fold increase over basal levels at 5 min into stimulation.

Concomitant with the decrease in PI radioactivity, a corresponding increase was measured for DAG which exhibited a much greater initial rise than did labeled PA, suggesting antigen stimulated receptor

mediated turnover of PI. Thus, while compd 48/80 and ionophore stimulated de novo PI synthesis, antigen stimulation induced a turnover. Unfortunately, the former investigations were carried out with Sprague-Dawley rat mast cells while the antigen-stimulated mast cells were derived from Wistar rats. Barring strain differences, it appears that mast cell PI metabolism may differ for different classes of secretagogues.

To date, this is the evidence that the PI response may play a role in mast cell activation-secretion. The PI response and the phospholipid methylation pathway are alike since they 1) are minor membrane lipids which undergo rapid turnover, 2) are associated with receptor stimulated activation, 3) may be involved in calcium mobilization, and 4) can serve as sources of AA. Their exact routes of metabolism and their physiological roles in mast cell degranulation require further experimentation.

Conceptual Model for Mast Cell Exocytosis

Delineation of events involved in mast cell release has evolved primarily from the impact of pharmacologic tools upon activation-secretion. For the most part, these tools have been enzyme inhibitors and antagonists and thus block events at different points along the presumed step-wise pathway of activation-degranulation.

In development of this model, we will focus upon the previously defined IgE dependent secretagogues including con A, specific antigen, and anti-IgE as well as polypeptide-basic types which include compd 48/80 and somatostatin. Though the conditions required to elicit mast cell release may be different for these two classes of secretagogues,

they all have one basic feature in common--they are all polyvalent. Sequentially, then, the first step in mast cell activation is ligand receptor binding and aggregation by the polyvalent secretagogue.

Use of the serine esterase inhibitors phenylmethylsulfonylfluoride (PMSF) or diisopropylfluorophosphate (DFP) to demonstrate blockade of histamine release as well as PC degradation suggests that activation of this enzyme may be an early event. When DFP is present during antigen challenge, no histamine release results. If it is removed prior to stimulation, release does occur, thus suggesting activation of this esterase by receptor aggregation. Since the inhibitors prevent PC degradation, serine esterase activation presumably occurs prior to activation of phospholipase A_2.

Phospholipid methylation which is stimulated selectively by IgE dependent secretagogues, but not polypeptide-base types, is inhibited by 3-deaza-SIBA or 3-deaza-adenosine. This results in the inhibition of $^{45}Ca^{2+}$ uptake as well as histamine release. In the absence of extracellular calcium, methylation, but not IgE-dependent release occurs, suggesting that phospholipid methylation may precede and/or modulate calcium influx.

A transient rise in cAMP has been measured during mast cell stimulation, but when the intracellular level is increased by theophylline or dibutyryl cAMP, antigen-induced $^{45}Ca^{2+}$ uptake and histamine release are inhibited but no effect was noted with A23187 as secretagogue. This suggests that cAMP may also be involved in calcium mobilization but its relationship to phospholipid methylation remains unclear. Fluctuations in cAMP levels may parallel these other events

but may be primarily involved in microtubule assembly-disassembly in preparation for cellular degranulation.

All these mentioned events precede both histamine and AA release. Activation of the calcium-dependent enzyme phospholipase A_2 may then occur. This enzyme is reportedly inhibited by mepacrine by some unknown mechanism of action, or by α-parabromoacetophenone (PBP) which modifies a histidine residue essential for its activity. Such inhibition blocks anti-IgE induced histamine release. Other evidence which suggests a role of phospholipase A_2 in mast cell secretion includes exogenous application of the enzyme which induces non-cytotoxic release and release of histamine from stimulated cells which is accompanied by release of [^{14}C]-AA from prelabeled phospholipids.

Oxidative metabolism of released AA <u>via</u> the cyclooxygenase pathway is exquisitely sensitive to inhibition by indomethacin and other non-steroidal anti-inflammatory agents while the lipoxygenase pathway remains undisturbed (Vane, 1971). However, indomethacin, at 10 μM, a concentration which completely suppresses oxidative conversion of AA, has no effect on the time course and amount of histamine released by A23187, con A or anti-IgE suggesting that AA metabolism is not necessary for or does not precede histamine release.

The inhibitors which block later events are the phenothiazines, believed to inhibit calmodulin and/or Ca/Pl-PK. These agents block degranulation by both IgE-dependent agents, polypeptide-base agents, as well as ionophoretic compounds. Since they block A23187 induced release of histamine without altering $^{45}Ca^{2+}$ uptake, it is assumed that the drugs act on processes which effect exocytosis.

Very little is known about protein phosphorylation-dephosphorylation, assembly-disassembly of cytoskeletal elements and generation of granule-plasma membrane fusogen events which are believed to comprise a final common pathway in mast cell release.

The goal of the present studies is to investigate the actions of two additional pharmacologic probes, hydrocortisone and 12-O-tetradecanoyl-phorbol acetate on events of mast cell activation-secretion coupling. This is the first characterization of either of these agents on purified, normal rat mast cells. Relevance of the hydrocortisone investigations arises particularly from the successful treatment of moderate to severe allergic asthma with anti-inflammatory steroids. The mechanisms by which anti-inflammatory steroids ameliorate symptoms remain unclear. Since this is a type I immediate hypersensitive reaction which involves mast cells, results of these present investigations with purified populations of mast cells exposed to hydrocortisone could lend valuable information concerning the mechanisms of action of anti-inflammatory steroids in cells which play a central role in allergic reactions.

In contrast, 12-O-tetradecanoyl-phorbol acetate, a prototypic active tumor promoting agent, is known to activate inflammatory type responses in several types of leukocytes. Its mechanism of action in certain types of these cells is becoming more clearly delineated. Thus, results of these investigations in mast cells could lend a new perspective on events of activation-secretion coupling.

Far-reaching goals of these investigations are to provide new approaches to pharmacologic modulation of components of the immune

system involved in allergic reactions and to gain further insight into basic exocytotic mechanisms since the mast cell is considered the model secretory cell.

CHAPTER TWO
INHIBITION OF IMMUNOGLOBULIN, BUT NOT POLYPEPTIDE-BASE, STIMULATED RELEASE OF HISTAMINE AND ARACHIDONIC ACID BY ANTI-INFLAMMATORY STEROIDS

Introduction

It is well established that glucocorticoids are potent anti-inflammatory agents effective in ameliorating the symptoms of immediate hypersensitivity and other allergic reactions (Lewis and Austen, 1981). Mechanisms of anti-inflammatory glucocorticoid action are not well delineated despite studies involving many varied cell types (Lewis and Piper, 1976; Gryglewski, 1976; Hong and Levine, 1976). Collectively, the data suggest that corticosteroids decrease the release of arachidonic acid from membrane phospholipids and thereby decrease prostaglandin generation without directly altering the enzymes involved in prostaglandin synthesis. Glucocorticoid induced inhibition of mast cell release may be related to the effectiveness of these agents in the treatment of asthma and other allergic reactions. The effects of glucocorticoids on mast cell release of both histamine and arachidonic acid have not been clearly delineated.

To clarify biochemical mechanisms of anti-inflammatory steroid action upon events in mast cell activation-secretion coupling, we conducted investigations on the effects of hydrocortisone and other glucocorticoids on the secretion of histamine and arachidonic acid and its metabolites. We report here that anti-inflammatory glucocorticoids

selectively inhibit release of histamine and [1-^{14}C]-arachidonic acid (AA) and its metabolites. Only IgE-like secretagogue induced release is steroid sensitive. Stimulation of both [1-^{14}C]-AA and histamine release by IgE independent secretagogues such as compd 48/80, somatostatin, and the calcium ionophore, A23187, is not altered by steroid pretreatment.

Materials and Methods

Ovalbumin Sensitization

Male or female Sprague-Dawley rats, 300-400g (random-bred colony, University of Florida, Gainesville, FL) were injected i.m. with 0.5 ml of a saline suspension containing 10 µg ovalbumin (Miles Research Products, Elkhart, IN) and 20 mg of $Al(OH_3)$ (Amphogel, Wyeth, Philadelphia, PA). Eight to ten days later a booster of the same dose was administered i.m. Animals were decapitated and the sensitized mast cells collected four days after the booster injection.

Mast Cell Preparation

Rat mast cells were obtained by lavage similar to the method of Sullivan et al., 1975. Briefly, animals were decapitated and injected intraperitoneally and intrathoracically with freshly prepared buffer containing 25 mM PIPES, 0.4 mM $MgCl_2$, 5 mM KCl, 10 mM NaCl, 0.1% (w/v) BSA or gelatin, 5.6 mM glucose, and 10 U/ml heparin in distilled water, pH 7.4. Trunks of the animals were massaged 100 times, the abdominal and thoracic cavities opened, the cell suspensions recovered and delivered to 50 ml polypropylene centrifuge tubes. Cells were washed and resuspended in the above buffer at one animal equivalent of cells per ml. Each ml of cells was carefully layered over 2 ml of buffer

containing 23% metrizamide (Accurate Chemical and Scientific Corp., Westbury, NY). Mast cells were purified by passage through the metrizamide layer during centrifugation at 180xg for 8 min as described by Yurt et al., 1977 and Coutts et al., 1980. Average yields were 1-1.5 x 10^6 mast cells/rat. Trypan blue exclusion (Sullivan et al., 1975) was used to assess cell viability (average >97%) and toluidine blue staining was used as a purity criterion (average approximately 90%).

Passive Sensitization

Purified mast cells were suspended in RPMI 1640 culture medium (5 x 10^5 cells/ml) (Flow Laboratory McLean, VA) containing 150 U/ml penicillin and 150 µg/ml streptomycin, 4 mM glutamine, 5% fetal calf serum, 200 ng/ml monoclonal mouse IgE anti-DNP antibody (Miles Research Laboratories, Elkhart, IN) and the cells incubated for 90 min.

Pretreatment of Mast Cells with Steroids

Hydrocortisone, as well as the other steroids tested, were dissolved in ethanol at a concentration of 1 mM then diluted to the appropriate final concentration in the above RPMI 1640 medium. Cells were suspended in the steroid-RPMI 1640 culture medium and incubated at 37°C with 5% CO_2 for 1 hr for acute steroid effects and 18-21 hr (overnight) for long-term effects. Cells were washed in fresh culture medium prior to [1-^{14}C]-AA incorporation or in the PIPES buffered salt solution prior to histamine release experiments.

Histamine Release and Measurement

Cells were suspended in the PIPES buffered salt solution containing 1 mM $CaCl_2$ to give 1 x 10^5 cells/0.45 ml. Aliquots of 0.45 ml were

dispersed to polypropylene tubes and histamine release initiated by addition of 50 µl of secretagogue. Histamine release by IgE dependent agents was carried out in the presence of 50 µg/ml bovine brain extract enriched in phosphatidylserine. Incubations were carried out for indicated times at 37°C with gentle agitation and release terminated by addition of 1 ml cold buffer then centrifugation at 5000xg for 10 min at 4°C. In each assay, a separate group of tubes was used for the determination of total cellular histamine by adding 0.4N $HClO_4$ prior to incubation. Supernatant histamine was determined by the o-phthaldialdehyde spectrophotofluorometric procedure (Shore et al., 1959) as modified by Anton and Sayre (1969) and Siraganian (1976). Percent histamine released was calculated as follows: ng supernatant histamine/ng total cellular histamine x 100.

Incorporation and Release of [1-^{14}C]-AA and Its Metabolites

Purified mast cells were suspended in the RPMI 1640 culture medium (described above) with [1-^{14}C]-AA (0.3 µCi/ml) then incubated for 60 min at 37°C. Cells were washed twice with RPMI 1640 medium and once with PIPES buffered salt solution containing 0.1% BSA as described previously (Crews et al., 1981). Cells were resuspended in PIPES buffered salt solution and allowed to equilibrate at 37°C for 30 min. Release of [1-^{14}C]-AA and its metabolites was carried out as described above for histamine. Total release was quantitated by liquid scintillation spectroscopy.

Measurement of ^{45}Ca Uptake

The method of Foreman et al. (1977) was used for the measurement of $^{45}Ca^{2+}$ flux. Briefly, purified mast cells were suspended in Tyrode's

solution, 2.5 x 10^5 cells/50 µl. One hundred microliters of versilube F50 silicone oil (General Electric Corp.) were placed in the bottom of a 400 µl microfuge tube and 40 µl Tyrode's solution containing $^{45}Ca^{2+}$ (5.5 µCi/ml) followed by 10 µl secretagogue in Tyrode's solution were layered on the oil and warmed to 37°C. Five minute incubations were initiated by addition of 50 µl mast cells and incubations halted by centrifugation in excess of 10,000xg for 30 sec in a Beckman B microfuge. Cell pellets were recovered by slicing off the microfuge tube tips, and vigorously shaking in vials with an aqueous solution of Triton X-100 (1%, v/v). $^{45}Ca^{2+}$ was assessed by addition of Liquiscint (National Diagnostics) to the solubilized pellets and counting in a liquid scintillation spectrometer.

Chemicals and Reagents

All steroids used were purchased from Sigma (St. Louis, MO) as were compd 48/80, con A, and somatostatin. The calcium ionophore, A23187, was purchased from Calbiochemical (LaJolla, CA). All other chemicals used were reagent grade. Radioligands were purchased from Amersham, [1-^{14}C]-AA, 60 mCi/mmole, and $^{45}Ca^{2+}$, 40 mCi/mg calcium.

Statistical Analysis

Data are expressed as the mean ± S.E.M. The Student's t-test was used to evaluate the differences between two means for significance. The criterion for significance was $p<0.05$. The EC_{50} (effective concentration for 50%) was calculated by probit analysis (Goldstein, 1964).

Results

To characterize the effects of glucocorticoids upon release of histamine and [1-^{14}C]-AA and its metabolites by rat mast cells, purified mast cells were incubated with hydrocortisone (3×10^{-6} M) overnight and then stimulated with known secretagogues. Hydrocortisone pretreatment markedly inhibited both histamine and [1-^{14}C]-AA release stimulated by concanavalin A (con A), the antigen ovalbumin (OA) and anti-immuglobulin E antibody (anti-IgE) (fig. 1). Histamine and [1-^{14}C]-AA release were inhibited to a similar extent. For example, hydrocortisone treatment inhibited anti-IgE stimulated histamine and [1-^{14}C]-AA by 83.7 ± 4.1% and 76.6 ± 1.5%, respectively. In contrast to IgE-like secretogogues, hydrocortisone pretreatment did not alter the release of either histamine or [1-^{14}C]-AA stimulated by the polypeptide somatostatin, compd 48/80 or the calcium ionophore, A23187 (fig. 2). Hydrocortisone pretreatment does not significantly alter the total amounts of mast cell histamine or incorporation of [1-^{14}C]-AA (Heiman and Crews, 1984). Since AA and its metabolites have been implicated in the mechanism of histamine release, we examined the effects of exogenous AA on hydrocortisone's inhibition of con A stimulated histamine release. Exogenous AA did not reverse hydrocortisone's inhibition of con A stimulated release. Con A (10 µg/ml) released 28.5 ± 0.8% and 28.2 ± 1.0% total cellular histamine in control cells with and without AA (1 µM), respectively. Cells treated for 18 hr with hydrocortisone released 13.4 ± 0.8% and 11.8 ± 0.2% total cellular histamine with and without exogenous AA (1 µM), respectively. These results indicate that hydrocortisone

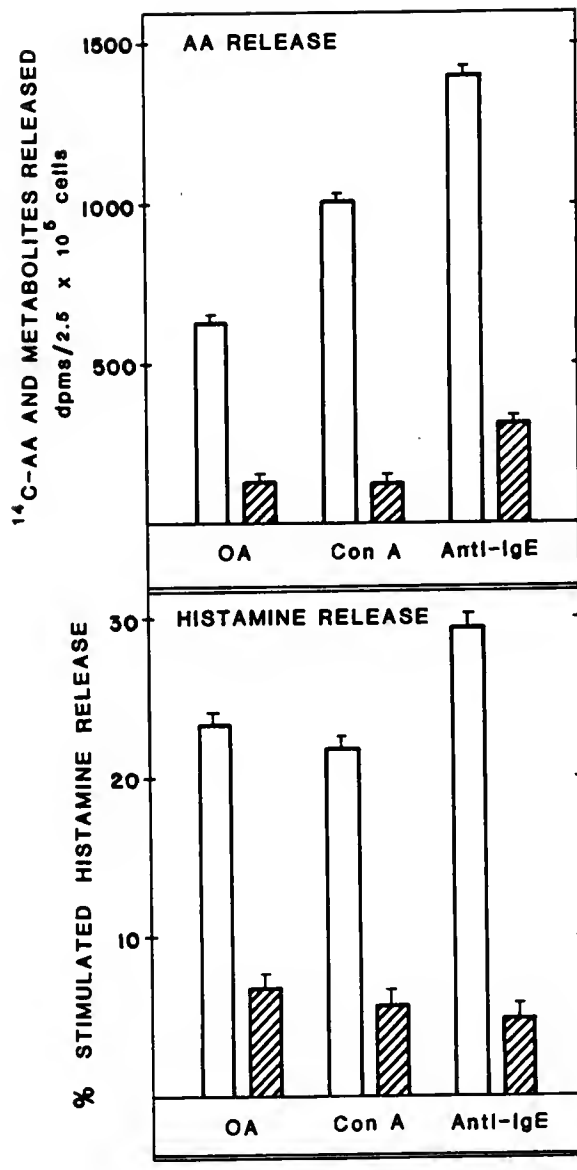

Figure 1. Effect of hydrocortisone pretreatment on the release of histamine and [1-^{14}C]-AA and its metabolites during stimulation with IgE-like secretagogues. Cells were stimulated for 30 min by addition of indicated secretagogue. Final stimulant concentrations were: OA, 100 µg/ml; con A, 3 µg/ml; anti-IgE, 1:1000 dilution. Spontaneous histamine release ranged from 8-12.9% and spontaneous [1-^{14}C]-AA release from 219 to 431 dpms/2.5 x 10^5 cells; these have been subtracted from values depicted. Open bars indicate untreated cells, striped bars represent hydrocortisone pretreated cells. Each bar represents a typical mean ± S.E.M. of triplicates repeated on at least two other occasions. All hydrocortisone treated groups were significantly different from controls at $p<0.05$ as assessed by Student's t-test.

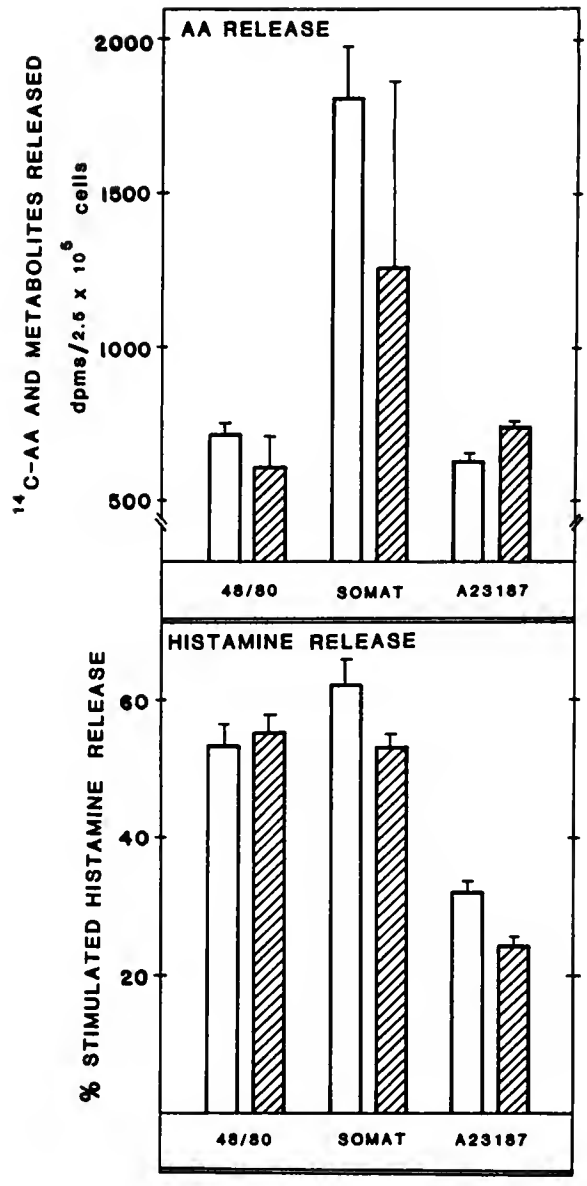

Figure 2. Effect of hydrocortisone pretreatment on histamine and [1-^{14}C]-AA release during stimulation with IgE-independent secretagogues. Cells were stimulated for 15 min with compd 48/80, 1 µg/ml; somatostatin, 100 µg/ml; A23187, 3 µm/ml. Spontaneous histamine release ranged from 3.8 to 9.0% and spontaneous [1-^{14}C]-AA release ranged from 128-210 dpms/2.5 x 10^5 cells; these have been subtracted from data shown. Open bars represent untreated cells and striped bars the hydrocortisone pretreated cells. Each bar represents a typical mean ± S.E.M. of triplicates repeated on at least two other occasions.

inhibits IgE-like release, i.e. anti-IgE, antigens and con A, but not polypeptide-base or ionophore stimulated release.

To further characterize the actions of glucocorticoids on mast cell release, time courses and concentration response curves were determined for IgE-like and IgE-independent secretagogues. Treatment with hydrocortisone (3×10^{-6} M) overnight slowed both the initial rate of histamine release and the total amount of histamine released by con A, but did not affect the rate or amount of histamine released by compd 48/80 (fig. 3). Results very similar to those for con A were obtained with anti-IgE as the stimulant (data not shown). To determine if the inhibition of stimulated release was due to a decrease in sensitivity or a loss of responsiveness, complete concentration responses curves were performed. Response curves for ovalbumin and compd 48/80 indicated that hydrocortisone pretreatment markedly decreased release at all ovalbumin concentrations tested, but did not alter compd 48/80 induced release (fig. 4). The ovalbumin ED_{50} for histamine release (untreated 1.14 ± 0.3 µg/ml; hydrocortisone, 1.19 ± 0.2 µg/ml) was not shifted by hydrocortisone pretreatment. For [1-^{14}C]-AA release, the untreated ED_{50} for ovalbumin was 0.70 ± 0.1 µg/ml, that for hydrocortisone treated cells was 0.43 ± 0.2 µg/ml. These data suggest that hydrocortisone treatment selectively decreases maximal release of histamine and [1-^{14}C]-AA initiated by an IgE-like secretagogue stimulation, but not by IgE-independent types of stimulation. These results, also suggest a close correspondence between release of histamine and [1-^{14}C]-AA and its metabolites.

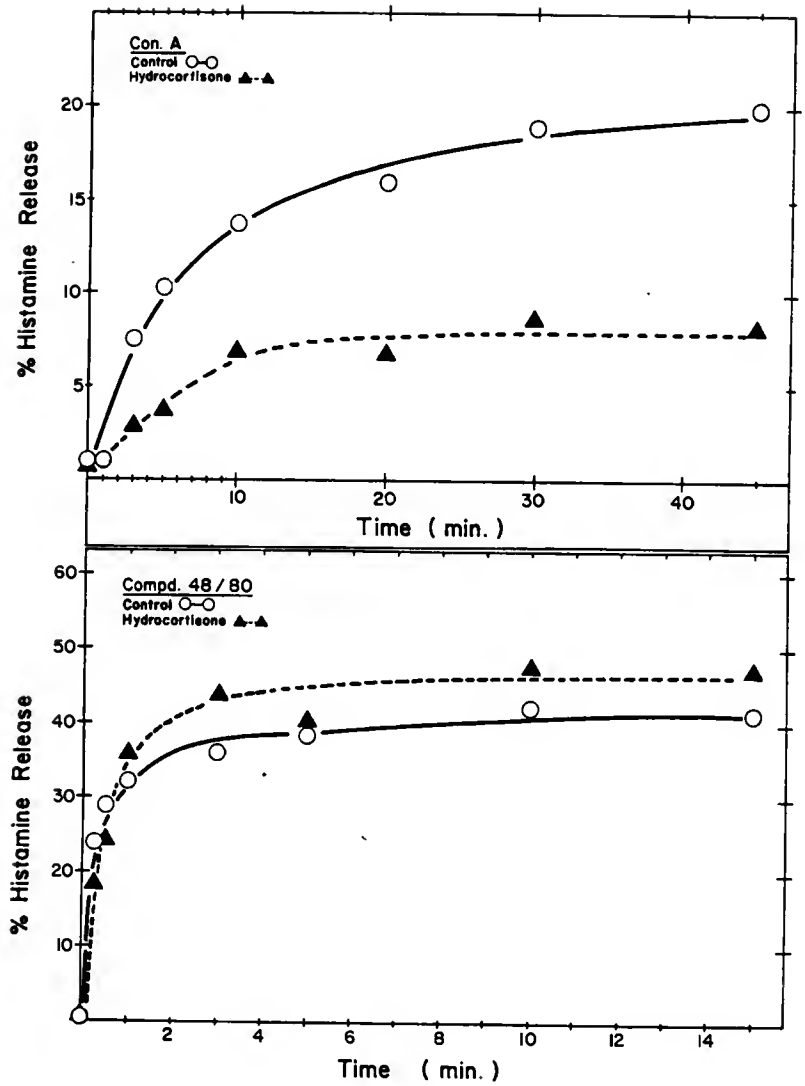

Figure 3. Time course of con A and compd 48/80 induced histamine release. Cells were preincubated with and without hydrocortisone (3×10^{-6} M) for 21 hr and then stimulated to release histamine by the addition of either con A (10 µgm) in presence of 50 µgm/ml bovine brain extract or compd 48/80 (3 µgm/ml). Histamine release is calculated as a percent of total releasable mast cell histamine. Each point represents the mean for triplicate determinations from one of two experiments of similar design.

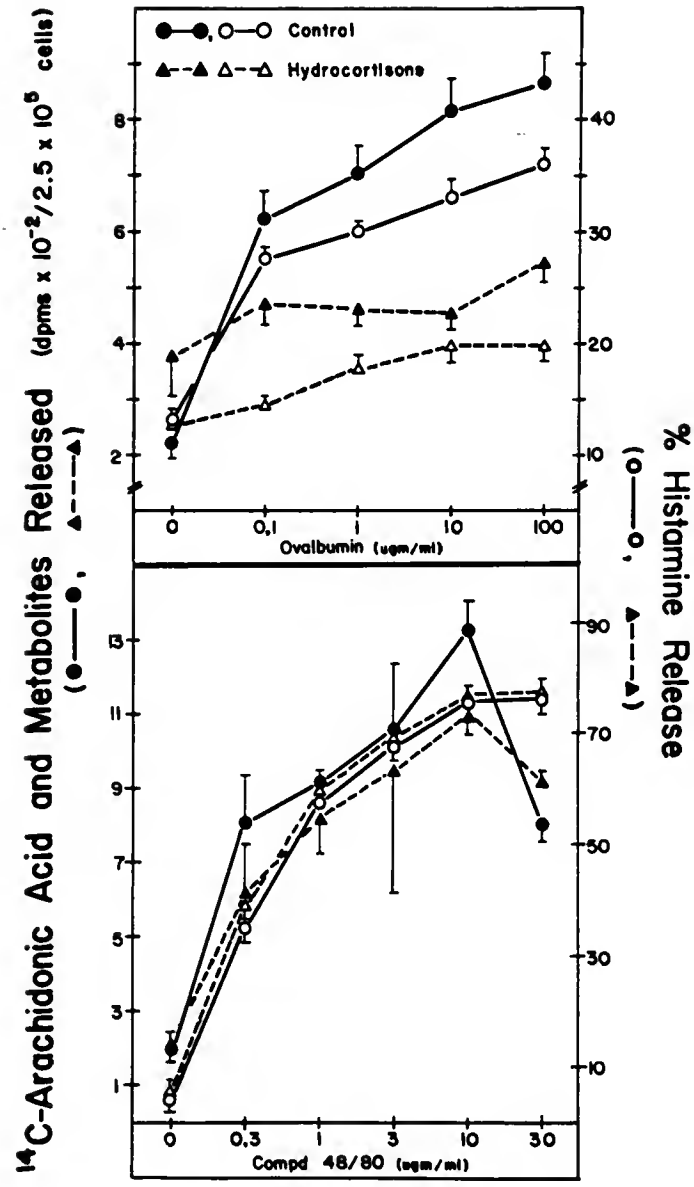

Figure 4. Dose dependent [1-^{14}C]-AA and histamine release by antigen ovalbumin (OA) and compd 48/80. Cells were stimulated to release histamine for 30 min by the addition of OA and 15 min for compd 48/80. Each point represents the mean ± S.E.M. of triplicate determinations from one experiment. Results are typical of those from at least two other experiments of similar design.

To determine the duration of hydrocortisone pretreatment required for inhibition of release initiated by IgE-like secretagogues, mast cells were incubated for 24 hr with various times of exposure to hydrocortisone (3×10^{-6} M). No inhibition of histamine release was found after 1, 3 or 9 hr of treatment, but following 12 hr of exposure to hydrocortisone, there was a significant decrease in histamine release (fig. 5). This time dependent inhibition slowly increased to approximately 95% inhibition after 24 hr of pretreatment. A similar time dependent inhibition was noted for IgE-like release of [1-^{14}C]-AA and its metabolites. Such a delay in the onset of inhibition is suggestive that induction of a protein may be involved in the mechanism of the glucocorticoid action on mast cell secretion.

To explore the possibility that steroid-induced protein synthesis is involved in the inhibition of IgE-mediated histamine release, we incubated cells with various concentrations of cycloheximide and actinomycin D, protein synthesis inhibitors with different sites of action. These agents alone inhibited histamine release (data not shown); therefore, we could not demonstrate reversal of the glucocorticoid inhibitory effect with either cycloheximide or actinomycin D. In rat thymus cells, glucocorticoid-receptor complex translocation to the cell nucleus is temperature dependent, i.e. this migration is halted at 4°C (Mosher et al., 1971; Wira and Munck, 1974). Thus, we studied the effects of temperature on the actions of hydrocortisone in our system. Preincubation of mast cells for 21 hr at 4°C or 37°C did not alter subsequent con A induced release at 37°C (fig. 6). As shown previously, 21 hr of preincubation with

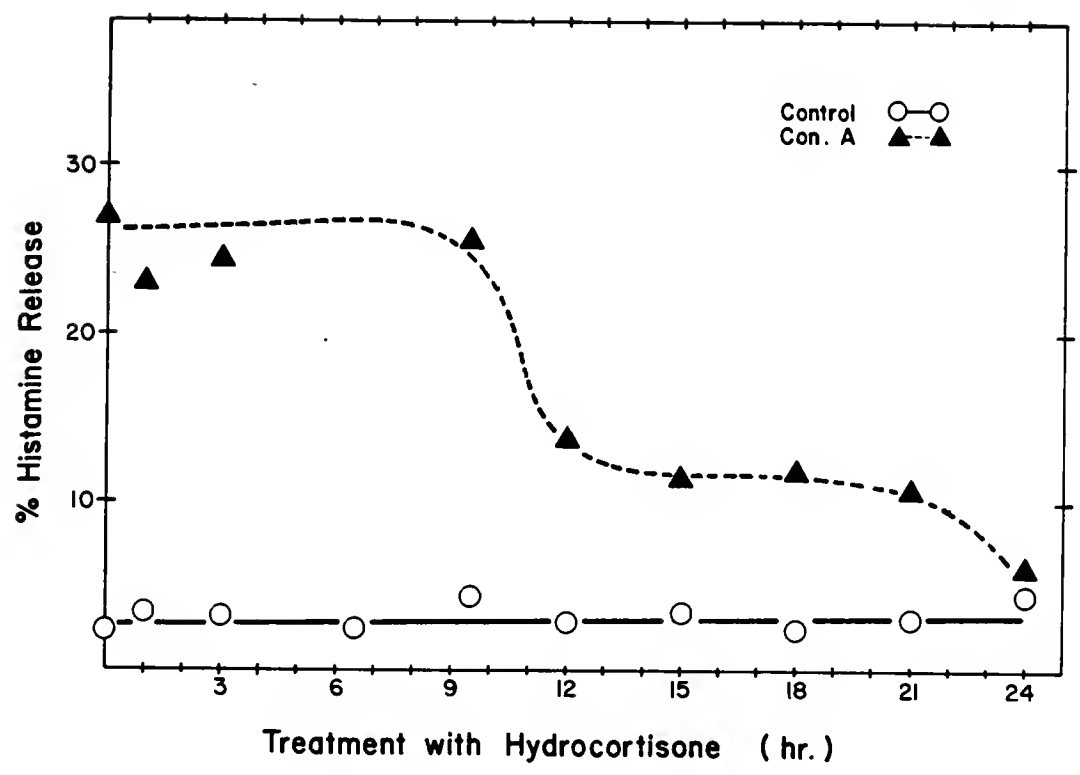

Figure 5. Time dependent inhibition of histamine release by hydrocortisone. Shown is an experiment typical of two other experiments. All cells were incubated for 24 hr, but drug-treated cells were exposed to hydrocortisone (3 x 10^{-6} M) for the number of hours indicated. Open circles indicate spontaneous histamine release and closed triangles indicate con A (10 μgm/ml) stimulated release of histamine from treated cells. Con A stimulated release from control cells ranged from 28-30% throughout the 24 hr time course. Points represent the mean of two determinations. Duplicates varied by less than 10%.

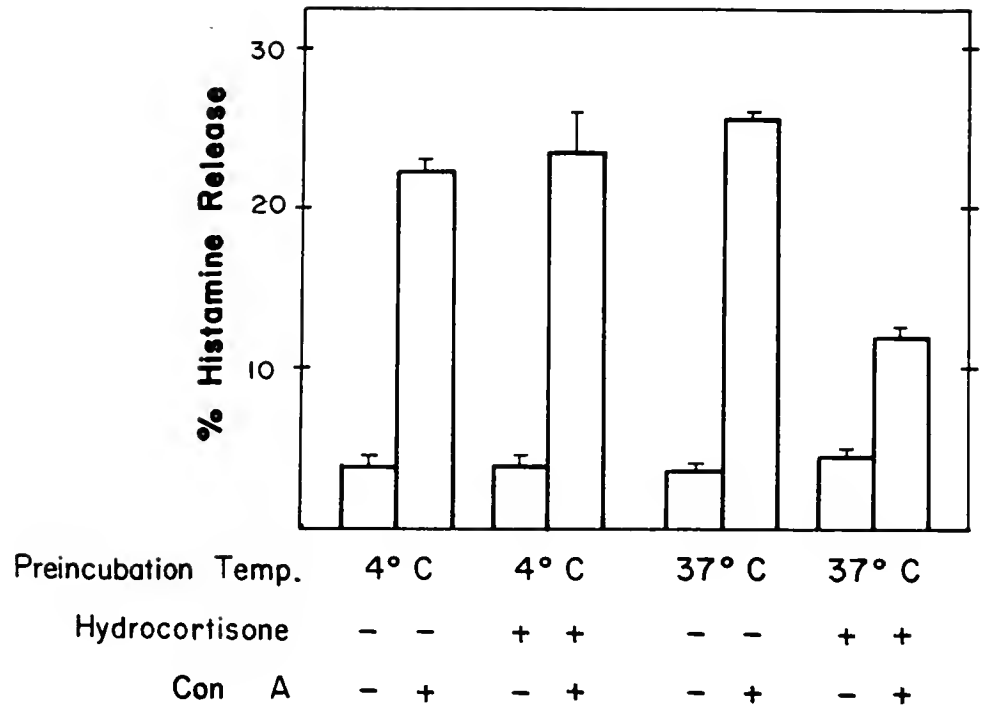

Figure 6. Temperature dependent inhibition of histamine release by hydrocortisone. Mast cells were preincubated with and without hydrocortisone (3×10^{-6} M) at 4°C or 37°C for 21 hr. Cells were washed, slowly warmed to 37°C and challenged to release histamine for 30 min by addition of con A (10 µg/ml). Bars represent the mean percent of total mast cell histamine ± S.E.M. for three separate determinations.

hydrocortisone at 37°C markedly inhibited con A induced histamine release. However, 21 hr of preincubation at 4°C with hydrocortisone did not alter subsequent con A induced histamine release at 37°C (fig. 6).

To delineate the specificity of the glucocorticoid induced inhibition of release, we incubated mast cells with several different types of steroids. We found that fluocinolone, dexamethasone, and hydrocortisone inhibited con A stimulated histamine release in a dose-dependent manner following a 21 hr preincubation (fig. 7). Steroid IC_{50} values were 1.4×10^{-8} M, 3.4×10^{-8} M, and 3.6×10^{-7} M, respectively. This order of potency parallels both their *in vivo* anti-inflammatory potencies (Gilman *et al.*, 1980) and their affinities for the glucocorticoid cytosolic receptor (Dausse *et al.*, 1977). Cells were also treated overnight with high concentrations (10^{-5} M) of the non-glucocorticoid steroids, estradiol, and testosterone. Stimulated histamine release by con A from untreated cells was 17.0 ± 0.7% while cells treated with testosterone and estradiol released 15.4 ± 0.8% and 17.4 ± 0.4% total histamine, respectively. Using fixed concentrations of anti-inflammatory steroids, inhibition was also obtained for release of [1-^{14}C]-AA and its metabolites (table 1). Thus, only anti-inflammatory steroids inhibit mast cell release by IgE-like secretagogues.

The importance of calcium in exocytotic mechanisms has been well documented for IgE-like mast cell secretagogues (Foreman *et al.*, 1977; Baxter and Adamik, 1978). To further investigate the site of action of glucocorticoids, we treated cells with hydrocortisone for 21 hr and

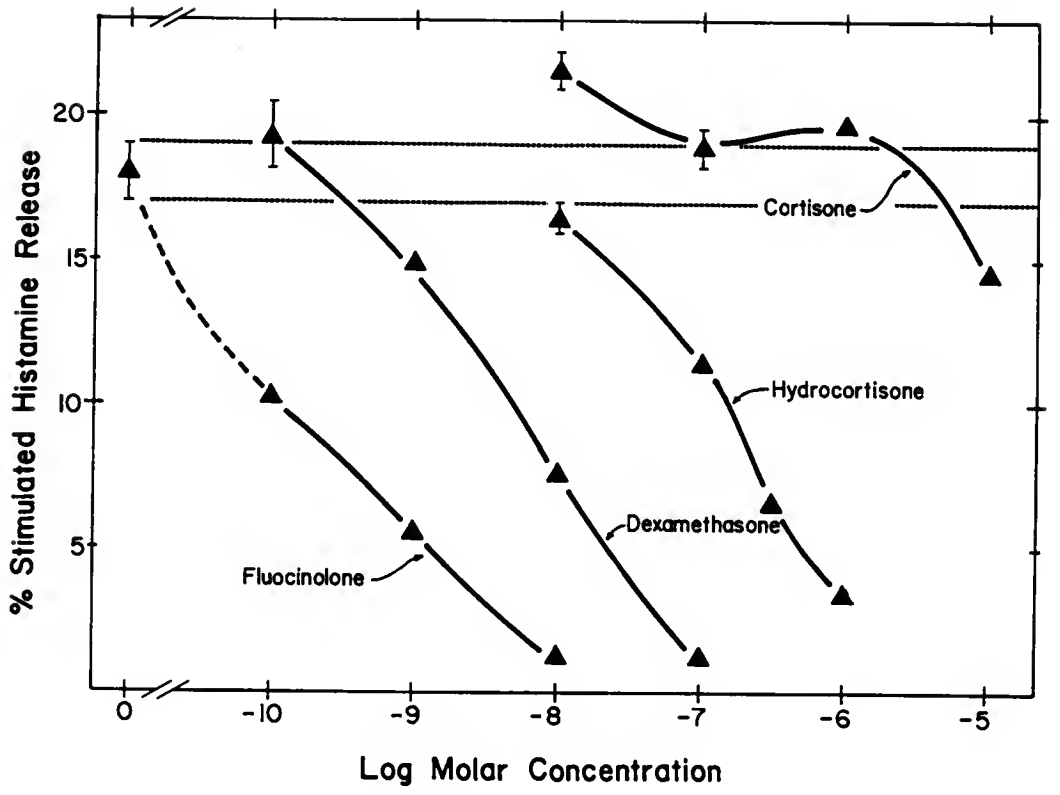

Figure 7. Inhibition of mast cell histamine release by anti-inflammatory steroids. Steroids were dissolved in ethanol (which comprised 0.1% of the pretreatment incubation volume) and cells incubated with indicated concentrations for 21 hr. Cells were washed, resuspended and challenged to release histamine for 30 min by addition of con A (10 μg/ml). Points represent the mean percent of total histamine released ± S.E.M. for three separate determinations.

TABLE 1

INHIBITION OF [1-^{14}C]-AA RELEASE BY ANTI-INFLAMMATORY STEROIDS

Treatment	dpms [1-^{14}C]-AA released/10^6 cells	Histamine (% Released)
None	14,817	43
Fluocinolone (10^{-8} M)	447	9.7
Dexamethasone (10^{-8} M)	819	10
Hydrocortisone (10^{-6} M)	875	12.3

Cells were pretreated for 18 hr with indicated concentrations of steroids, washed, resuspended in PIPES buffered salt solution containing 1 mM $CaCl_2$ and 1% BSA, 0.45 ml aliquots containing 2.5 x 10^6 cells dispensed and release initiated by addition of con A (10 µgm/ml) in presence of 50 µgm/ml BBE. Incubations at 37°C were continued for 30 min. Subtracted background [1-^{14}C]-AA releases averaged 2686 dpm/10^6 cells, and subtracted background histamine releases averaged 5.7%. Values represent the mean of duplicates from an experiment replicated with similar outcomes on two other occasions.

then stimulated histamine and [1-^{14}C]-AA and metabolite release with the calcium ionophore, A23187, which artificially induces calcium flux. Ionophore induced release was not significantly inhibited by hydrocortisone treatment (fig. 8), suggesting that the secretory and AA release processes following the influx of calcium are not altered by glucocorticoid treatment. Since release stimulated by IgE-like secretogogues is known to depend upon extracellular calcium (Baxter and Adamik, 1978), we studied the effect of hydrocortisone treatment on the influx of $^{45}Ca^{2+}$. Stimulation of rat mast cells with antigen caused a rapid influx of calcium which was completed 5 min after addition of the secretagogue. Pre-treatment with hydrocortisone for 18 hr markedly reduced calcium influx induced by con A, antigen, and anti-IgE (fig. 9). These results suggest that glucocorticoids inhibit plasma membrane calcium flux and thereby selectively inhibit secretagogues dependent on extracellular calcium, i.e. IgE-like secretagogues.

Discussion

Antigen and anti-IgE antibodies are thought to stimulate release from rat mast cells by cross-linking IgE-Fc receptor complexes (Ishizaka and Ishizaka, 1978). Con A, in a manner similar to antigen, appears to act by cross-linking IgE bound to the cell surface (Siraganian and Siraganian, 1975). These IgE-like secretagogues have several properties which distinguish them from the polypeptide-base secretagogues, e.g. somatostatin and compd 48/80. IgE-like secretagogues are dependent upon extracellular calcium, potentiated by phosphatidylserine (Baxter and Adamik, 1978), stimulate phospholipid

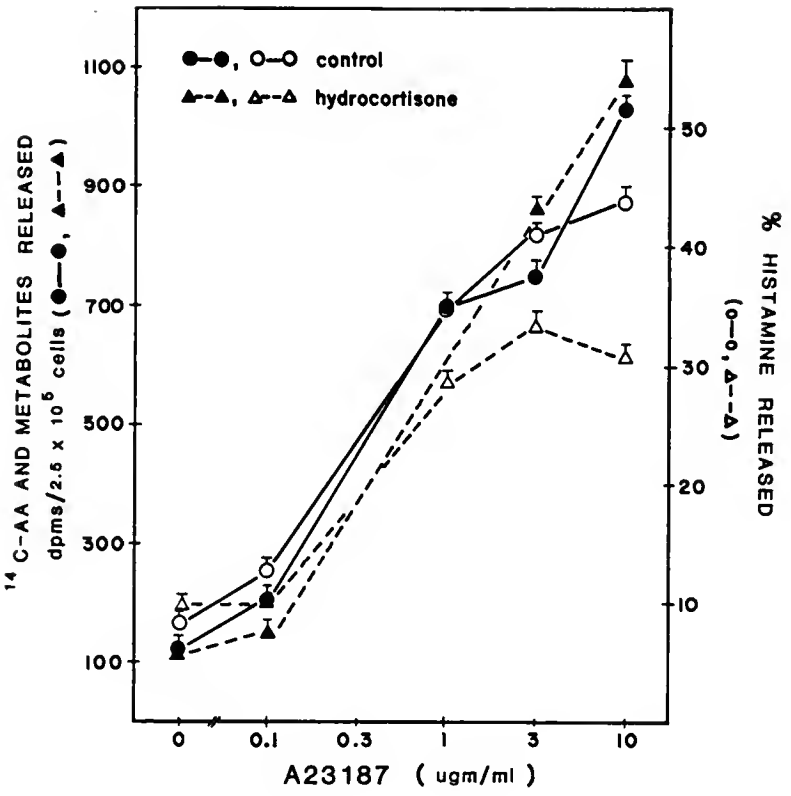

Figure 8. Effects of hydrocortisone treatment on A23187-induced release of histamine and [1-^{14}C]-AA and its metabolites. Cells were treated as described in the legend of figure 4, then resuspended and challenged to release histamine by addition for 15 min of indicated concentrations of the calcium ionophore, A23187. Points represent the mean percent of total histamine released ± S.E.M. for three determinations.

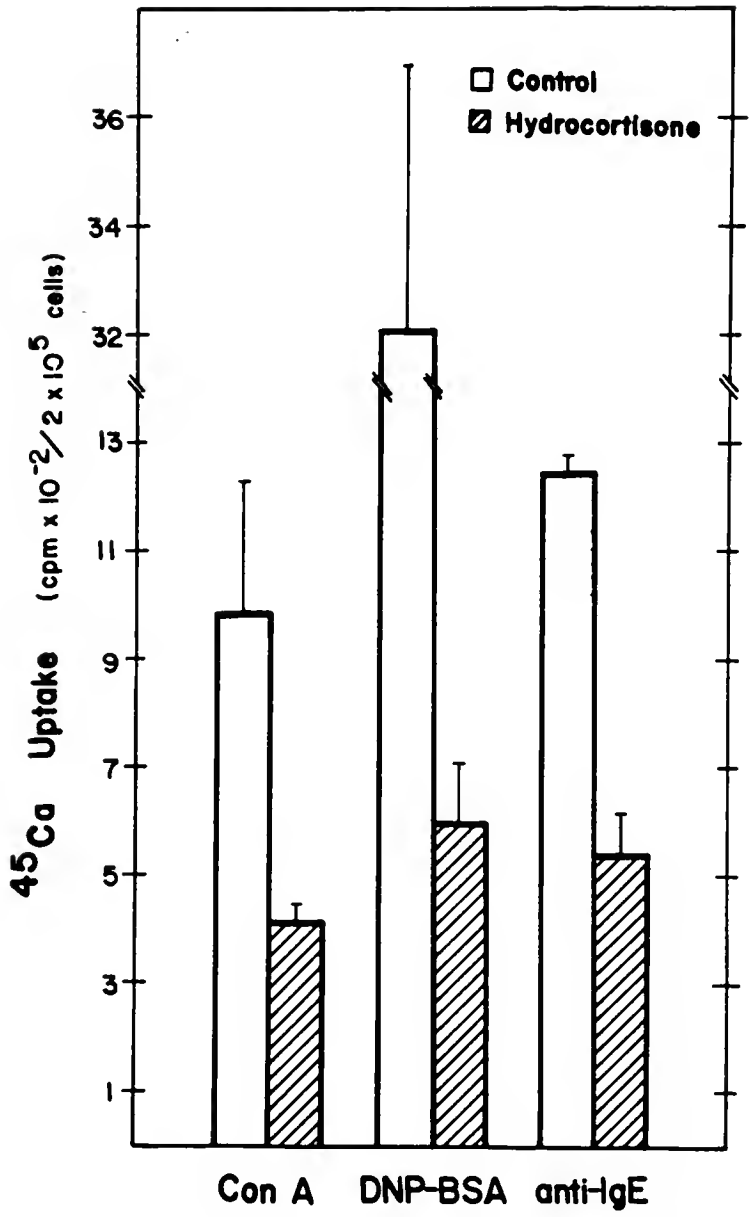

Figure 9. Effect of hydrocortisone pretreatment on IgE-dependent $^{45}Ca^{2+}$ uptake. Mast cells were preincubated with and without hydrocortisone (3×10^{-6} M) then challenged for 5 min with the following secretagogues in the presence of 5.5 µCi/ml $^{45}Ca^{2+}$: con A 10 µg/ml, DNP-BSA 0.1 µg/ml, anti-IgE 1:100 dilution. Bars represent the mean stimulated uptake of $^{45}Ca^{2+}$ cpm/2 x 10^5 cells ± S.E.M. for three separate determinations. Background $^{45}Ca^{2+}$ values were 300 ± 17 cpm/2 x 10^5 cells. All hydrocortisone treated groups were significantly different from controls at $p<0.05$ as assessed by Student's t-test.

methylation (Hirata et. al., 1979) and are inhibited by methylation inhibitors (Crews et al., 1981). Somatostatin and compd 48/80 do not have any of these requirements or effects. These polypeptide-base secretagogues appear to act by releasing internal stores of calcium (fig. 10). Somatostatin and compd 48/80 have been suggested to act on the same membrane receptor (Theoharides et al., 1981). They are both strong secretagogues which typically release more than 40-50% of the total cellular histamine as compared to IgE-like secretagogues which have a maximal response of about 20-30% total cellular histamine. The strength of stimulation may play a role in the glucocorticoid affect. However, submaximal concentrations of compd 48/80 which release approximately 30% of total cellular histamine are not altered by glucocorticoid treatment (fig. 4). These findings, along with the differential effects of the glucocorticoids, suggest that release of histamine and arachidonic acid and its metabolites induced by the IgE-Fc receptor complex occurs by triggering mechanisms different from those of somatostatin-48/80 induced release and that glucocorticoids act by selectively uncoupling IgE-mediated release (fig. 10).

Glucocorticoids can act by membrane stabilization and/or induction of the synthesis of specific proteins (fig. 10). The latter is thought to involve the binding of glucocorticoids to a cytosolic receptor, and formation of a complex which translocates to the nucleus and increases synthesis of specific mRNA (Grody et al., 1982). Cytosolic glucocorticoid receptors have been demonstrated in mast cells (Daeron et al., 1982). Other studies have indicated that the translocation of the glucocorticoid-receptor complex is temperature dependent (Mosher et

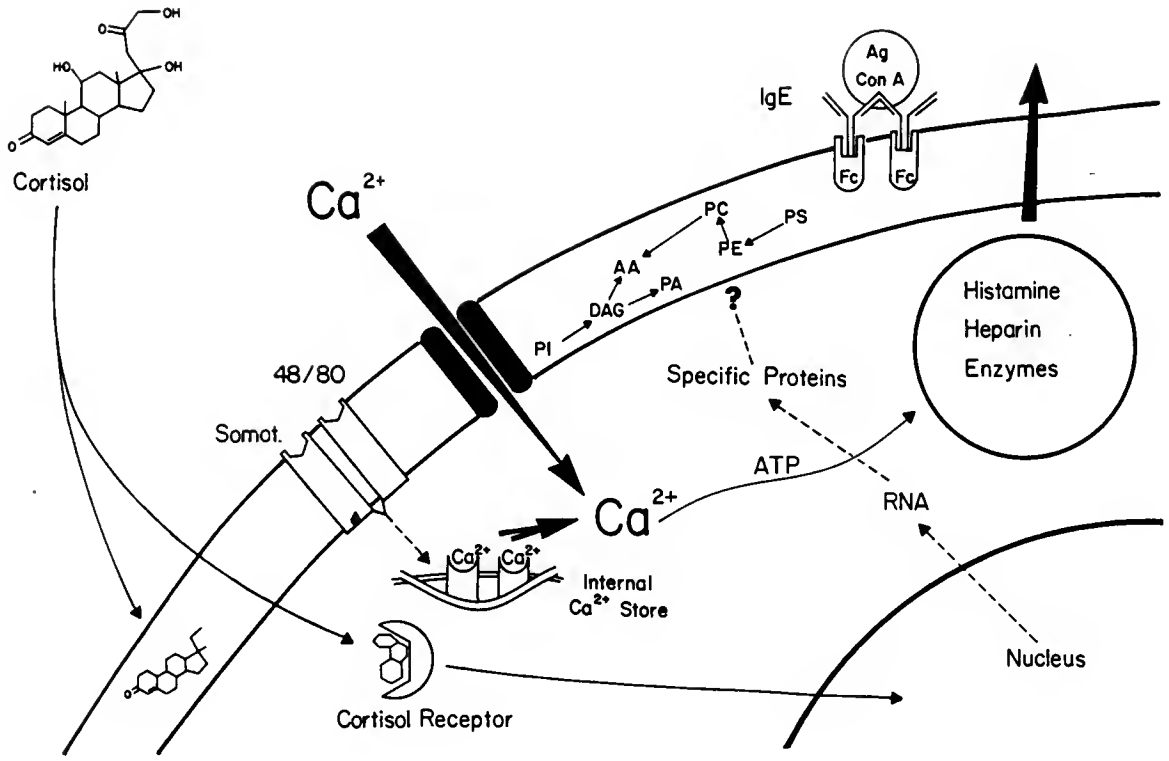

Figure 10. Schematic diagram of possible sites of cortisol action on mast cell exocytosis. Cortisol could act through a cytoplasmic receptor which when activated migrates to the nucleus and induces the synthesis of specific proteins. A membrane effect is also possible. The specific proteins synthesized appear to interfere with the coupling of the IgE receptors to the influx of calcium. Phospholipid methylation (i.e. PE-->PC) and/or phosphatidylinositol turnover (i.e. PI-->DAG-->PA) have been implicated in the coupling of IgE receptors to calcium influx and the release of arachidonic acid (AA). Phospholipid methylation may be inhibited by steroid treatment (Daeron et al., 1982). Somatostatin and compd 48/80 appear to release internal stores of calcium. Therefore, these secretagogues are not inhibited by glucocorticoids. Abbreviations: IgE, immunoglobulin E; Ag, antigen; con A, concanavalin A; somat., somatostatin; 48/80, compd 48/80; Fc, cell surface receptor to which IgE binds; ATP, adenosine triphosphate; RNA, ribonucleic acid.

al., 1971; Wira and Munck, 1974). The following data suggest that glucocorticoids inhibit rat mast cell histamine release by inducing the synthesis of specific proteins. Membrane stabilization occurs acutely, whereas we have demonstrated that the inhibition of histamine and arachidonic acid release by glucocorticoids requires several hours of treatment. Inhibition of con A (IgE-like) induced histamine release by glucocorticoids is temperature dependent, as is glucocorticoid-receptor complex translocation. Various glucocorticoid inhibitory potencies reported here parallel their in vivo anti-inflammatory potencies (Gilman et al., 1980) and their affinities for the cytosolic receptor (Dausse et al., 1977). Thus, it is likely that induction of a specific protein is an important component of the glucocorticoid action on rat mast cells.

Studies on rat mast cells (Hirata et al., 1979) and rat basophilic leukemia cells (Crews et al., 1981; McGivney et al., 1981) have suggested that the stimulus-secretion coupling sequence is as follows: antigen cross-linking of IgE, stimulation of phospholipid methylation, influx of calcium, activation of phospholipase initiating fusion of granule and plasma membranes (fig. 10). Our findings indicate that glucocorticoids selectively inhibit IgE-mediated release by reducing IgE stimulated calcium flux. Studies in mouse mast cells have suggested that glucocorticoids alter anti-IgE stimulated histamine release by inhibiting phospholipid methylation (Daeron et al., 1982). These studies indicated that steroid treatment does not change IgE-Fc receptor number or affinity. Other investigators studying anti-inflammatory steroid action have found that steroids induce the

synthesis of a phospholipase inhibitory protein in rat leukocytes (macrocortin) (Blackwell et al., 1980), rabbit neutrophils (lipomodulin) (Hirata et al., 1980), and mouse mast cells (Daeron et al., 1982). Our finding that the glucocorticoid inhibitory effect upon arachidonic acid release occurs only for IgE-like secretagogues and is not overcome by exogenous AA is inconsistent with a general inhibition of phospholipase. Several subtypes of phospholipase may exist, with only the IgE-receptor associated form of the enzyme exhibiting susceptibility to anti-inflammatory pretreatment. It has now been proposed that the majority of lipomodulin secreted from neutrophils is phosphorylated (therefore inactive) then split into several smaller molecules the smallest of which is a 16,000 dalton protein (the size of macrocortin) also generated by lymphocytes and called IgE-suppressive factor or glycosylation inhibiting factor (Vede et al., 1983). This suggests, then, that a fragment of lipomodulin is involved in the selective formation of IgE-suppressive factors which modulate T cell subset responses to immunologic stimuli. A similar protein, generated by glucocorticoid treated mast cells could contribute to the selectivity of the inhibition induced. Alternatively, glucocorticoids may also synthesize a phospholipid methyltransferase inhibitory protein. Additional studies are necessary to delineate the mechanisms of glucocorticoid specificity.

Whatever the mechanism of glucocorticoid action, our findings indicate that glucocorticoids selectively inhibit IgE-mediated release, i.e. anti-IgE, antigen and con A, but do not alter IgE-independent stimulation of histamine and arachidonic acid release by somatostatin,

compd 48/80 or the calcium ionophore, A23187. The precise mechanisms of steroid action in asthma and other allergic reactions are unknown. Responsiveness differs for various types of allergic reactions (Patterson, 1979). Our finding suggests that steroid treatment may reduce allergic reactions mediated by antigen-IgE, but not reactions stimulated by peptides and toxins which act through other receptors.

CHAPTER THREE
CHARACTERIZATION OF THE EFFECTS OF PHORBOL ESTERS ON RAT MAST CELL SECRETION

Introduction

Phorbol esters (PEs), the most potent tumor promoting agents in the mouse skin bioassay, also cause a variety of biological responses in diverse cell types (Blumberg, 1980). These include lymphocyte mitogenesis (Touraine et al., 1977; Abb et al., 1979), platelet aggregation and serotonin release (Mufson et al., 1979; Zucker et al., 1974), PMN superoxide anion production (Lehrer and Cohen, 1981), interleukin 2 production by mouse EL4 thymoma cells (Kraft et al., 1982), as well as histamine release from a mixed population of human leukocytes (Schleimer et al., 1980).

We became interested in studying the effects of PEs alone and in conjunction with various secretagogues for several important reasons. First, it is well recognized that when applied to the skin, PEs elicit signs of acute inflammation, mediators of which are released by mast cells (Boutwell, 1974). Second, skin has been shown to contain specific binding sites for PEs (Delclos et al., 1980), and skin contains a large number of mast cells. Recent studies have suggested that PEs may specifically activate a calcium/phospholipid dependent protein kinase (Ca/PL-PK) (Yamanishi et al., 1983; Castagna et al., 1982; Ashendel et al., 1983). An investigation of the action of PEs on mast cell secretion could provide a valuable tool for delineating the

role of this protein kinase in mediator release since experimental results with stimulated mast cells have shown that during secretion there is a rapid calcium-dependent phosphorylation of certain protein bands (Sieghart et al., 1978; Theoharides et al., 1980; Wells and Mann, 1982).

In the purified mast cell system, receptor-mediated stimulation can occur via two major classes of secretagogues which we call IgE-like and polypeptide-base as well as other types of secretagogues (Crews and Heiman, 1984; Lagunoff et al., 1983). We define IgE-like as those secretagogues which appear to activate mast cells by interacting with bound immunoglobulin E. We call somatostatin and compd 48/80 polypeptide-base secretagogues since these agents appear to release histamine through a cell surface receptor which is not IgE. Properties which distinguish the two classes of secretagogues which we investigate are: IgE-like agents (antigen, anti-IgE, concanavalin A) require extracellular calcium, are potentiated by phosphatidylserine (Baxter and Adamik, 1978), stimulate phospholipid methylation, and are sensitive to glucocorticoid pretreatment when used to stimulate mast cell secretion (Crews and Heiman, 1984). Polypeptide-base secretagogues (somatostatin, compd 48/80) do not require extracellular calcium and do not respond to the conditions described for IgE-like secretagogues.

Our interest in the Ca/PL-PK centers in the similarities for its activation and mast cell secretion, namely calcium mobilization and enhancement of release by PS. Abundant occurrence of Ca/PL-PK and its endogenous substrate proteins in the particulate fraction of human

neutrophils suggests that protein phosphorylations by this enzyme may be involved in membrane associated neutrophil functions. This hypothesis was further strengthened by the demonstration, in these same studies, that phosphorylation was inhibited by trifluoperazine, an agent which inhibits neutrophil chemotaxis, aggregation and degranulation (Helfman et al., 1982). Trifluoperazine is also known to inhibit rat mast cell secretion elicited by antigen, compd 48/80 and by the calcium ionophore A23187 (Douglas and Nemeth, 1982).

We report here that 1) PE tremendously potentiates A23187 stimulated release of histamine and arachidonic acid, 2) extracellular calcium is required for release in the presence of PE and A23187, 3) histamine release by IgE-like secretagogues is potentiated by PS and TPA, 4) structure activity relationships for the effects of various PE analogs on mast cell histamine release suggest a single mechanism of action, perhaps mediated by a single receptor, 5) TPA either alone or with A23187 significantly increased phosphorylation of mast cell protein(s), and 6) mast cells have low levels of Ca/PL-PK.

Materials and Methods

Mast cell preparation. Rat mast cells were obtained by lavage similar to the method of Sullivan et al. (1975). Briefly, animals were decapitated and injected intraperitoneally and intrathoracically with freshly prepared buffer containing 25 mM PIPES, 0.4 mM $MgCl_2$, 5 mM KCl, 10 mM NaCl, 0.1% (w/v) BSA or gelatin, 5.6 mM glucose, and 10 U/ml heparin in distilled water, pH 7.4. Trunks of the animals were massaged, the ventral wall reflected, the cell suspensions withdrawn and delivered to 50 ml polypropylene centrifuge tubes. Cells were

washed and resuspended in the above buffer at one animal equivalent of cells per ml. Each ml of cells was carefully layered over 2 ml of 23% metrizamide (Accurate Chemical and Scientific Corp., Westbury, NY) also prepared in the above buffer without heparin. Mast cells were purified by passage through the metrizamide layer during centrifugation at 180xg for 8 min as described by Yurt et al. (1977) and Coutts et al. (1980). Average yields were 1-1.5 x 10^6 mast cells/rat. Trypan blue exclusion was used to assess cell viability (average >97%) and toluidine blue staining was used as a purity criterion (average approximately 90%).

Preparation of secretagogues. Ten times the final concentrations of all secretagogues were prepared in the PIPES buffered salt solution described above. In cases where IgE-like secretagogues were used, 500 µgm/ml phosphatidylserine (PS) was added to the buffer and sonicated for 2 min prior to addition of concanavalin A (con A) or sheep anti-rat IgE (anti-IgE).

PEs were dissolved in DMSO as stock solutions of 1 mg/ml and were diluted in buffer. During secretagogue induced release from mast cells, DMSO comprised .001% of the incubated volume and in vehicle treated control cells did not alter mediator release.

Histamine release and measurement. Cells were suspended in the PIPES buffered salt solution containing 1 mM $CaCl_2$ to give 1 x 10^5 cells/0.45 ml. Aliquots of 0.45 ml were dispensed to polypropylene tubes and histamine release initiated by addition of 50 µl of secretagogue. Histamine release by IgE dependent agents was carried out in the presence of 50 µgm/ml phosphatidylserine from bovine brain. Incubations were carried out for indicated times at 37°C with gentle

agitation and release terminated by addition of 1 ml cold buffer then centrifugation at 4000xg for 10 min at 4°C. In each assay, a separate group of tubes was used for the determination of total cellular histamine by adding 0.4N $HClO_4$ prior to incubation. Supernatant histamine was determined by the o-phthaldialdehyde spectrophotofluorometric procedure (Shore et al., 1959) as modified by Anton and Sayre (1969) and Siraganian (1976). Percent histamine released was calculated as follows: ng supernatant histamine/ng total cellular histamine x 100. Spontaneous histamine release, specified in each figure legend, has not been subtracted.

Phosphorylation of mast cell proteins. In a method similar to that employed by Wells and Mann (1982), cells were suspended in the PIPES buffered salt solution containing 1 mM $CaCl_2$ at 2 x 10^6 mast cells/ml and incubated at 37°C for 30 min with [^{32}P]orthophosphate, carrier free, at a concentration of 1 mCi/ml. Cells were then washed twice in 5 ml warmed buffer and the pelleted cells resuspended to a final cell density of 10^5 cells/50 µl. Fifty micro-liters of resuspended cells were delivered to warmed glass tubes and stimulation initiated by addition of 5 µl of secretagogue. Incubations were 60 sec and terminated by the addition of 50 µl of gel sample buffer containing 20% w/v glycine, 4% w/v SDS, 0.008% bromophenol blue and 10 µl mercaptoethanol/ tube. Tubes were placed in a boiling water bath for 3 min, cooled, sealed and stored overnight at -20°C prior to SDS-PAGE.

SDS-PAGE. Electrophoresis was carried out in a Bio-Rad dual slab cell. The resolving gel was 8% acrylamide (w/v) with an acrylamide:bis-acrylamide ratio of 29:1. Samples (50 µl) were applied

to wells cast in the stacking gel with a 15-slot comb and gels calibrated by applying the following MW standards from a commercial standards kit (Sigma): carbonic anhydrase, 29 kDa; egg albumin, 45 kDa; BSA, 66 kDA; β-galactosidase, 116 kDA. Slabs were stained with 0.2% Coomassie brilliant blue R, dried and autoradiographed using SAR-6 x-ray film (Kodak) at -70°C. To quantitate phosphorylation, autoradiograms were scanned with a densitometer (E-C Apparatus Corp.) and peak heights used to represent relative units of radioactivity (Ueda et al., 1973).

Measurement of Ca/PL-PK. Total, solubilized fractions of cerebral cortex, resident peritoneal cells (RPC), RPC-devoid of mast cells (RPC - mast cells; cells which form a band at the buffer-matrizamide interface) and mast cells were prepared according to the method described by Helfman et al. (1982). Washed RPCs were resuspended in 2 ml Hank's balanced salt solution (HBSS) and treated for 5 min at 4°C with 5 mM DFP. Cells were washed twice in cold HBSS, mast cell and RPC - mast cell fractions prepared as described above, pelleted and resuspended in 0.5 ml homogenization buffer (50 mM Tris-HCl, pH 7.5; 1% v/v β-mercaptoethanol; 2 mM EGTA; 0.1% v/v Triton X-100; 1 mM phenylmethylsulfonyl fluoride). Dissected cerebral cortex tissue was delivered into 5 volumes of homogenization buffer then homogenized by hand in a glass-teflon tissue homogenizer. Cortical homogenate served as positive Ca/PL-PK control in all experiments. All tissue preparations were then sonicated at 50 watts for 20 sec (Sonifier Cell Disruptor, Ultrasonics, Inc., Plainview, NY), stirred gently for 1 hr at 4°C, then centrifuged at 100,000xg for 60 min. The resulting

supernatants were used as the source of enzyme. Ca/PL-PK enzyme assay conditions were as follows. Total incubation volumes were 200 µl and contained 25 mM PIPES (pH 6.5), 10 mM $MgCl_2$, 250 µM EGTA, 300-500 µM $CaCl_2$, 1 nmol (containing 0.9 to 1.4 x 10^6 cpms of [Y-^{32}P]ATP), 40 µgm histone 1, 3-4 µgm supernatant protein in the presence or absence of 50 µgm phosphatidylserine. Reactions were carried out for 4 min at 30°C and halted by the addition of 4 ml trichloroacetic acid (TCA) containing 0.25% w/v sodium tungstate. Forty microliters of 1.625% BSA were added and tubes centrifuged at 2000 rpm (500xg) for 1 min. Supernatants were aspirated, pellets redissolved in 0.1 ml 0.5N NaOH then precipitated again with TCA-tungstate solution. Precipitates were washed in this manner three times, redispersed in 0.1 ml 0.5N NaOH and an aliquot counted after addition of 5 ml Liquiscint (National Diagnostics, Somerville, NJ).

Chemicals and reagents. All phorbol esters used were purchased from Sigma (St. Louis, MO) as were compd 48/80, con A, somatostatin, phosphatidylserine, and ATP. The calcium ionophore, A23187, was purchased from Calbiochemical (LaJolla, CA) and anti-IgE from Miles. Culture medium was purchased from Flow Laboratories and [1-^{14}C]-AA and [^{32}P]orthophosphate from Amersham. All other chemicals used were reagent grade.

Results

Time Course and Calcium Requirement for TPA Induced Histamine Release

The time course of TPA induced release of mast cell histamine in the presence and absence of subthreshold concentration of the calcium ionophore, A23187, is shown in figure 11. While A23187 (0.05 µg/ml) or

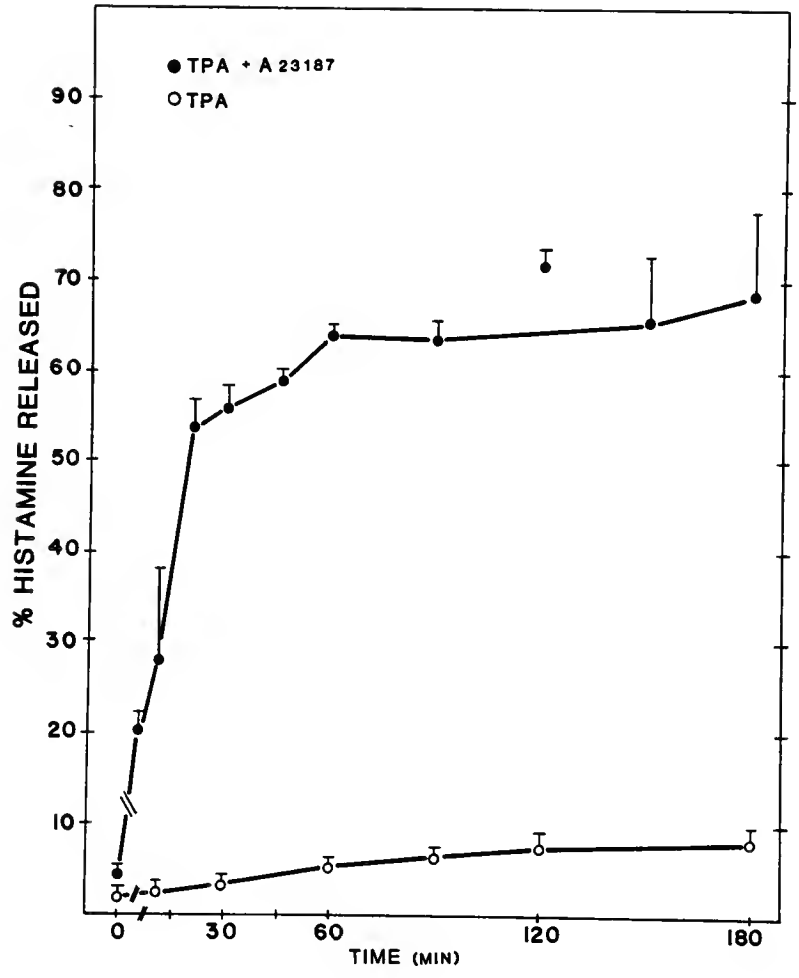

Figure 11. Time course for TPA induced histamine release. Purified rat mast cells were incubated with TPA, 10 ng/ml in the presence and absence of A23187, 0.05 µgm/ml for the indicated times at 37°C. Spontaneous histamine release was 4 ± 0.5% and has not been subtracted. A23187 alone released 6.6 ± 0.4%. Each point represents the mean percent histamine released ± SEM of triplicates from an experiment replicated with similar outcomes on two other occasions.

TPA (10 µg/ml) alone released less than 10% of total cellular histamine over the 3 hr time course, these agents acted synergistically to produce a significant release after 5 min. Histamine was linearly released for about 20 min, reaching a plateau of approximately 60% total histamine released at 30 min. After this initial release period, there was a slow gradual increase in histamine release reaching approximately 70-80% after 3 hr and up to 90-95% after 5 hr. To determine the role of extracellular calcium, cells were preincubated for 90 min in PIPES-buffered salt solution containing either 10^{-4} M EDTA or 2 mM calcium chloride then stimulated with TPA plus ionophore A23187 in the same medium. Cells preincubated then stimulated in presence of EGTA released only 6% histamine over spontaneous release (5%), while cells preincubated and stimulated in presence of calcium released 60% total cellular histamine.

Effect of TPA on Concentration Response of the Calcium Ionophore A23187

The calcium ionophore, A23187, is thought to stimulate histamine release by directly elevating free intracellular calcium levels. Concentration response curves for A23187 with and without TPA are depicted in figure 12. The histamine release elicited by ionophore with TPA was potentiated (shifted to the left) when compared with A23187 alone. The EC_{50} for the ionophore was approximately 832 ng/ml and was reduced to 56.3 ng/ml when TPA was present as a co-stimulant. When the extracellular calcium was removed, neither the co-stimulants TPA-A23187 nor A23187 alone stimulated release.

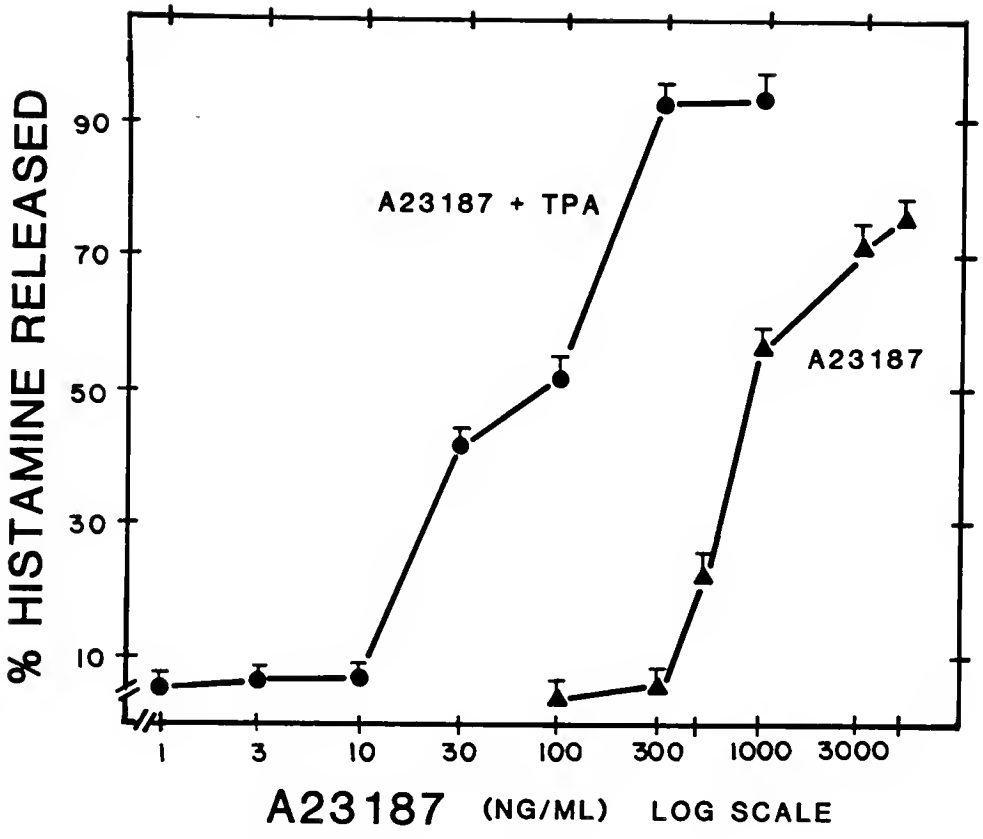

Figure 12. Effect of TPA on the dose-response of the calcium ionophore A23187. Purified rat mast cells were incubated at 37°C for 30 min with various concentrations of A23187 in the presence and absence of TPA, 10 ng/ml. Spontaneous histamine release which has not been subtracted was 4.1 ± 0.5%. Each point represents the mean ± SEM of triplicate determinations from one experiment. Results are typical of those from at least two other experiments of similar design.

Structure-activity Relationship for the Effect of Various PE Analogs on Mast Cell Histamine Release

Specificity of the PE effect was explored by examining the structure activity relationships for a series of five PEs. These studies indicated an order of potency: TPA > 4β-PDD > POE (Fig.13). Approximate EC_{50} values for the active PEs were: 5.4 ng/ml, 83.1 ng/ml, and 807 ng/ml, respectively. Agents inactive as tumor promotors, e.g. 4α-PDD and 4α-phorbol were inactive as releasers of histamine. Active-inactive isomeric pairs are suggestive of receptor specificity. In experiments of a similar design cells were loaded with [1-^{14}C]-AA prior to stimulation with TPA and subthreshold A23187. The maximal response of both histamine and [1-^{14}C]-AA release occurred at 3 ng/ml TPA with 32.1 ± 0.8% and 4421 ± 510 cpms/10^6 cells, respectively. Basal histamine release in presence of subthreshold A23187 was 4.7 ± 0.4% while [1-^{14}C]-AA release was 1528 ± 46 cpms/10^6 cells. Thus, co-stimulation with TPA-A23187 appears to activate concomitant histamine and AA release. Neither TPA alone, nor the inactive tumor promoter 4α-phorbol induced release of these mediators.

Effect of TPA on IgE-like Secretagogue Induced Histamine Release

To characterize the effect of TPA on mast cell histamine release, concentration response curves were assessed for anti-IgE (Fig. 14, top) and con A (Fig. 14, bottom). At all concentrations of anti-IgE and con A tested, inclusion of TPA increased histamine release. Although TPA increased con A stimulated histamine release, it did not significantly change the EC_{50} of con A for release, being 0.68 μgm/ml and 0.66 μgm/ml

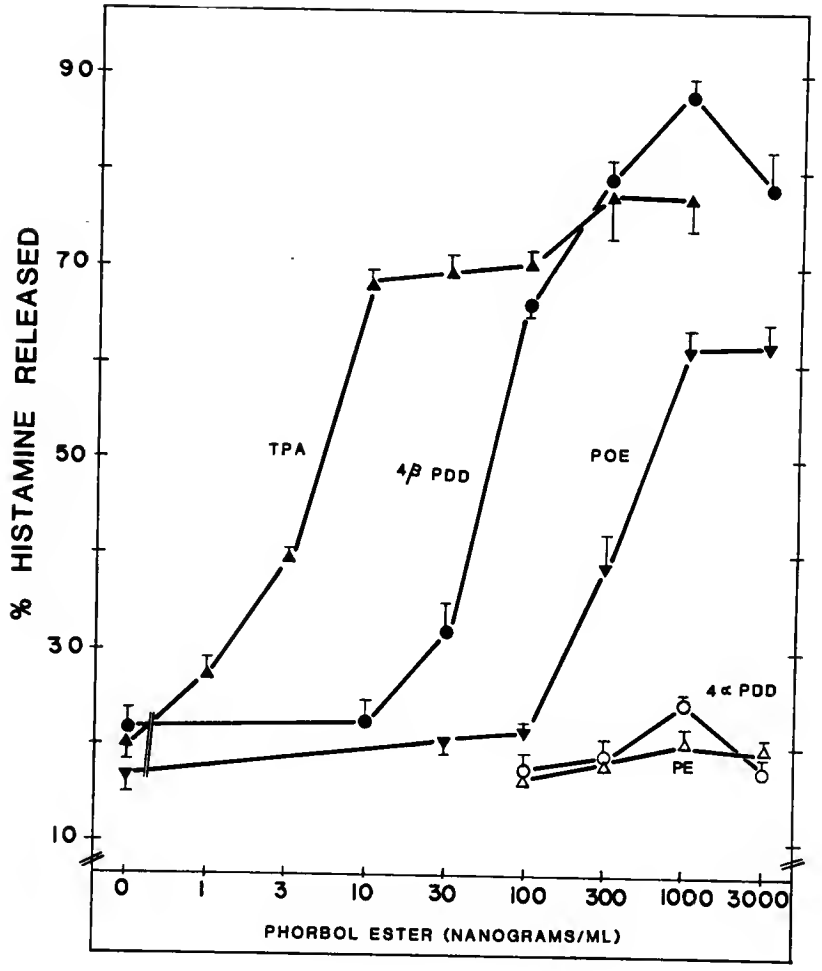

Figure 13. Specificity of the PE effect on mast cell histamine release. Purified rat mast cells were incubated at 37°C for 30 min with the indicated concentrations of PEs in the presence of 0.05 μmg A23187. Abbreviations are: TPA, 12-O-tetradecanoylphorbol-13-acetate; 4βPDD, 4β-phorbol 12β,13α-didecanoate; POE, phorbol 12-myristate 13-acetate 4-O-methylether; PE, 4α-phorbol. Background release in the presence of A23187 was 17 to 22% and has not been subtracted. Points represent the mean percent of total histamine released ± SEM for triplicate determinations from one experiment. Similar results were obtained in one other experiment of identical design.

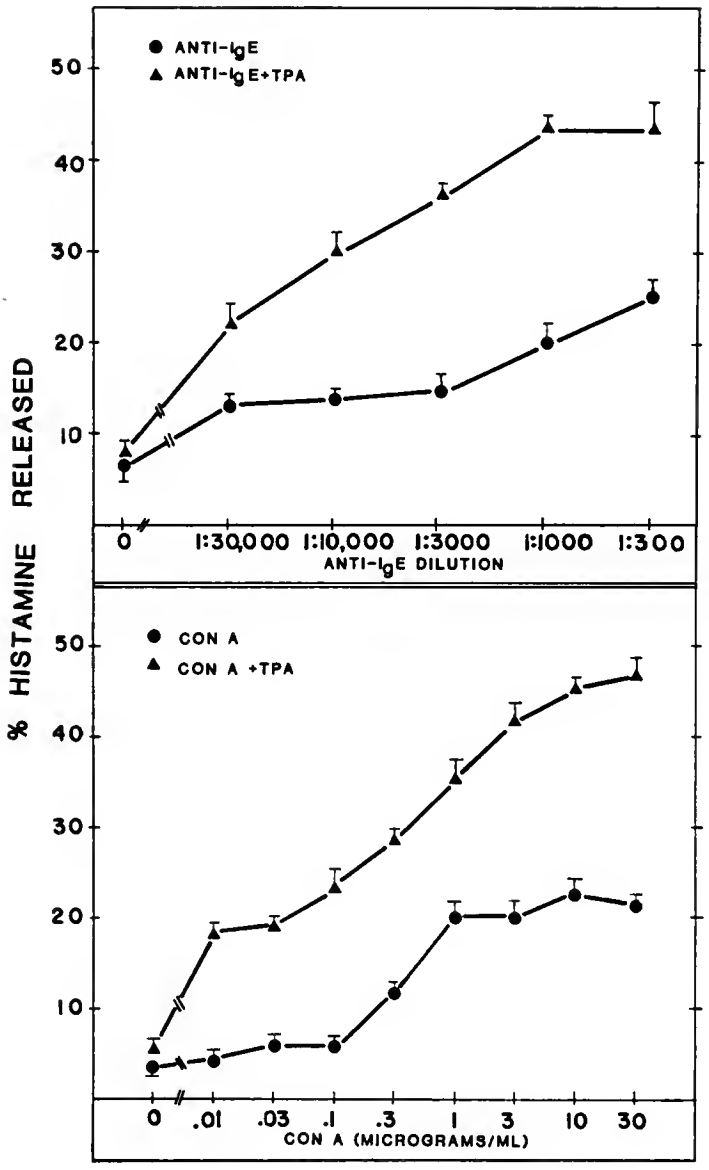

Figure 14. Effect of TPA on IgE-like secretagogue induced histamine release. Indicated concentrations of anti-IgE (top panel) and con A (bottom panel) in the presence of exogenous PS (50 µgm/ml) and in the presence and absence of TPA (10 ng/ml) were used to stimulate histamine release from purified rat mast cells. Spontaneous histamine release, not subtracted, was 7-7.6% for Anti-IgE experiments and 4-5.5% for con A experiments. Incubations were carried out at 37°C for 45 min. Each point represents the mean percent histamine released ± SEM of triplicates from one experiment. Results are typical of those from several other experiments of a similar design.

in the presence and absence of TPA, respectively. Thus, TPA exhibited a synergistic relationship with both con A and anti-IgE.

Enhancement of Stimulated Histamine Release by Phosphatidylserine and TPA

Since IgE-like secretagogues are known to be enhanced by exogenous PS, the interaction of TPA with PS was investigated. Con A alone elicited a very slight release response, which was greatly potentiated in the presence of PS (Table 2). A similar situation exists for anti-IgE. There was no significant dependence of peptide-base secretagogue induced release upon exogenous PS (Grosman and Diamant, 1975). When TPA was present with IgE-like secretagogues, but without PS, small increases in release were observed. This release was synergistically enhanced when both PS and TPA were present. The responses were more than additive even though maximal concentrations of PS and TPA were used.

Effect of TPA on Polypeptide-Base Secretagogue Induced Histamine Release

To determine the actions of TPA on compd 48/80 and somatostatin induced mast cell release, complete concentration response curves were done in the presence and absence of TPA (Fig. 15). In contrast to the IgE-like secretagogues, TPA and compd 48/80 co-stimulation was barely more than additive at 0.1 µgm/ml compd 48/80, and less than additive at all other concentrations. Release induced by TPA and/or somatostatin were less than additive at low concentrations and virtually identical at higher concentrations. Neither potentiation nor synergism was evident.

TABLE 1

EFFECT OF PS ON HISTAMINE RELEASE BY IgE-LIKE SECRETAGOGUES

Releasing Agent	Histamine Release (% Total Cellular Histamine)			
	Alone	PS (50 µgm/ml)	TPA (10 ng/ml)	PS + TPA
None	1.1 ± 0.1	3.1 ± 0.4	5.9 ± 0.4	7.6 ± 0.2
con A (10 µgm/ml)	3.8 ± 0.3	20.5 ± 0.9	13.0 ± 1.0	44.7 ± 1.5
anti-IgE (1:1000 dil.)	2.3 ± 0.7	14.0 ± 1.2	7.7 ± 0.3	33.0 ± 2.4

Purified mast cells were washed, equilibrated for 30 min, pelleted then resuspended in PIPES buffered salt solution containing 1 mM $CaCl_2$. 0.45 ml cells were aliquoted and stimulated by addition of 50 µl of indicated releasing agents. Incubations were carried out for 45 min at 37°C with secretagogue alone, and with PS and/or TPA. Values represent the mean percents of triplicate ± S.E.M. of total mast cell histamine. Similar results were obtained, in experiments of similar design, on two other occasions.

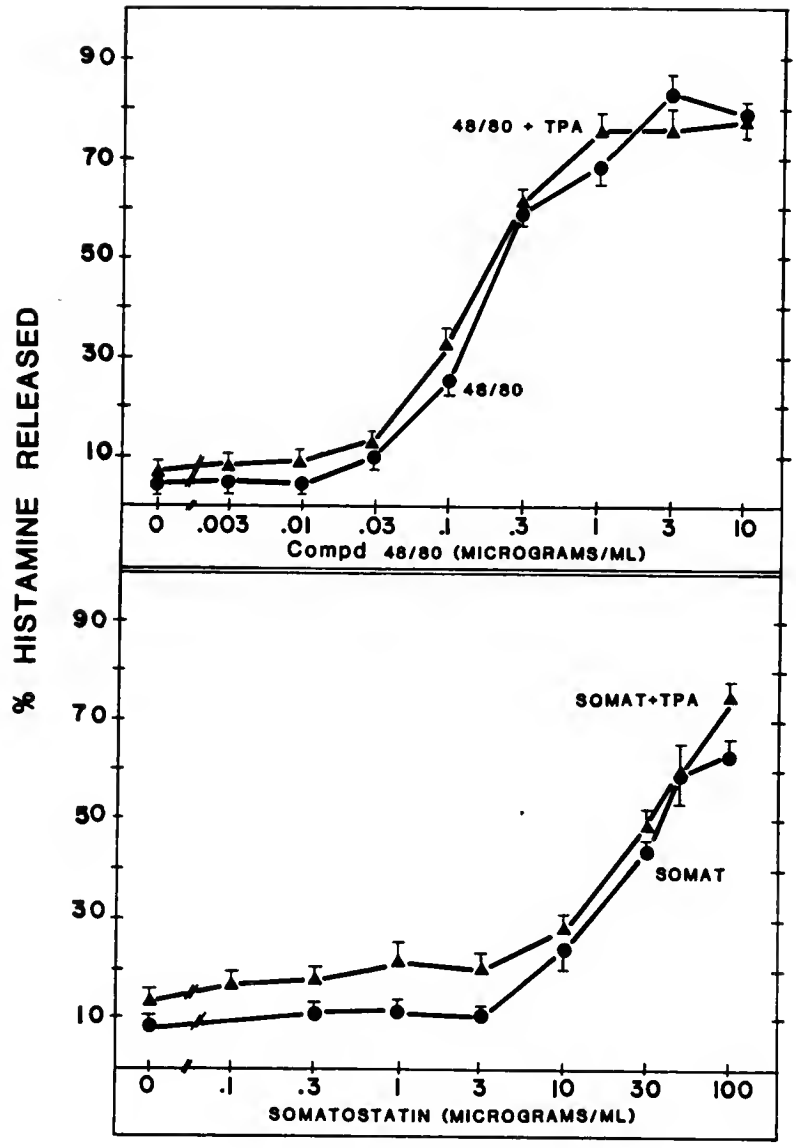

Figure 15. Effect of TPA on polypeptide-base secretagogue induced histamine release. Purified rat mast cells were incubated with the indicated concentrations of compd 48/80 (top panel) and somatostatin (bottom panel) in the presence and absence of TPA, 10 ng/ml, for 30 min at 37°C. Spontaneous release which has not been subtracted, was 4% for compd 48/80 alone, 5% with TPA (top panel) and 8% for somatostatin alone, 14% with TPA (bottom panel). Each point represents the mean percent histamine released ± S.E.M. of triplicates from one experiment. Results are typical of those from two other experiments of a similar design.

Effect of Secretagogues on Mast Cell Protein Phosphorylation

It is becoming increasingly evident that activation-release events of secretory cells are regulated, at least in part by protein phosphorylation-dephosphorylation. Recent studies suggest that PEs bind to and activate the Ca/PL-PK. Therefore, we investigated protein phosphorylation in mast cells. Autoradiographs of protein phosphorylations during 60 sec stimulation with con A, 48/80, anti-IgE, and TPA alone, suboptimal A23187 alone and TPA-suboptimal A23187 together are depicted in figure 16. Although a variety of proteins are phosphorylated, only a few are markedly changed during stimulation. We found significant changes in the phosphorylation of four major bands with apparent molecular weights of 78, 59, 55 and 48 kDa. The most consistent and largest changes occurred in the 48 kDa band (Fig. 16). Stimulation of mast cells with con A, anti-IgE, compd 48/80, TPA and TPA-A23187 at least doubled the phosphorylation of the proteins migrating with an apparent molecular weight of 48 kDa. Con A, compd 48/80, and TPA-A23187 stimulated histamine release corresponded with the increase in phosphorylation of the 48 kDa band. In contrast, anti-IgE and TPA alone stimulated much larger increases in phosphorylation of the 48 kDa band than histamine release. Although low dose ionophore (0.05 μgm/ml) had little effect on 48 kDa phosphorylation or histamine release (Figs. 16 and 17) secretion-inducing doses of A23187 (1 μgm/ml) stimulated phosphorylation of the 48 kDa band and other bands similar to con A, anti-IgE and compd 48/80. Thus, TPA alone and TPA-A23187 stimulate

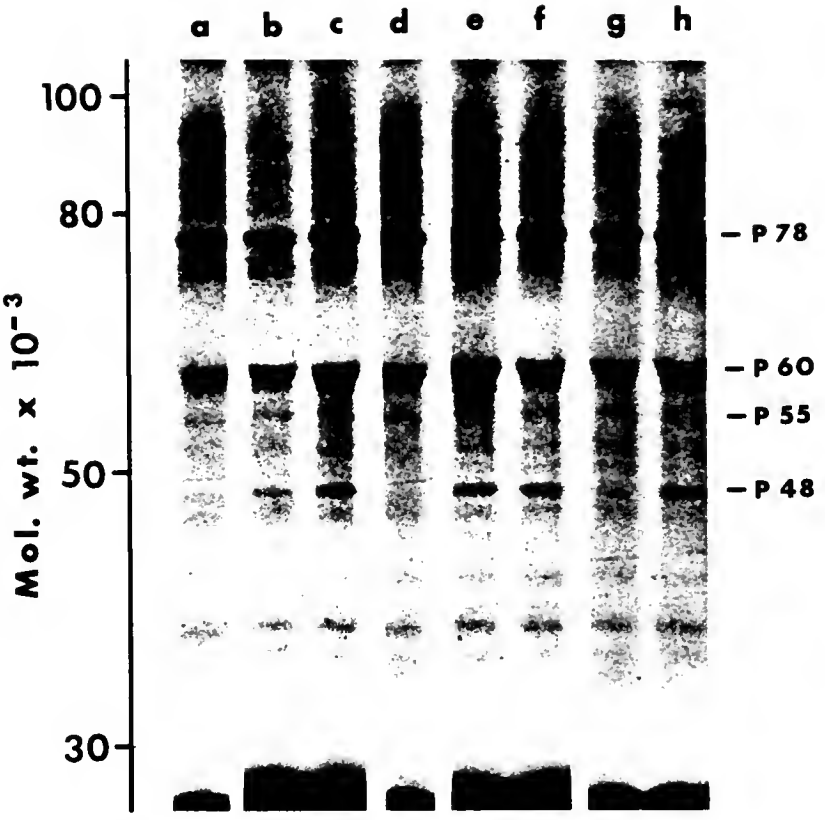

Figure 16. Autoradiographs of SDS-PAGE gels showing secretagogue induced phosphorylation of mast cell proteins. Incubation periods were for 60 sec and release of histamine from identically handled but unlabeled cells incubated with secretagogue for conventional 15 or 30 min are given in table 3. Secretagogues (concentrations shown in legend to figure 17) were: a) PS, b) con A + PS, c) anti-IgE + PS, d) none, e) compd 48/80, f) TPA, g) A23187, and h) TPA + A23187. Apparent molecular weights were determined by calibrating the gel with standard proteins of known molecular weight. Phosphorylated proteins are shown on the right.

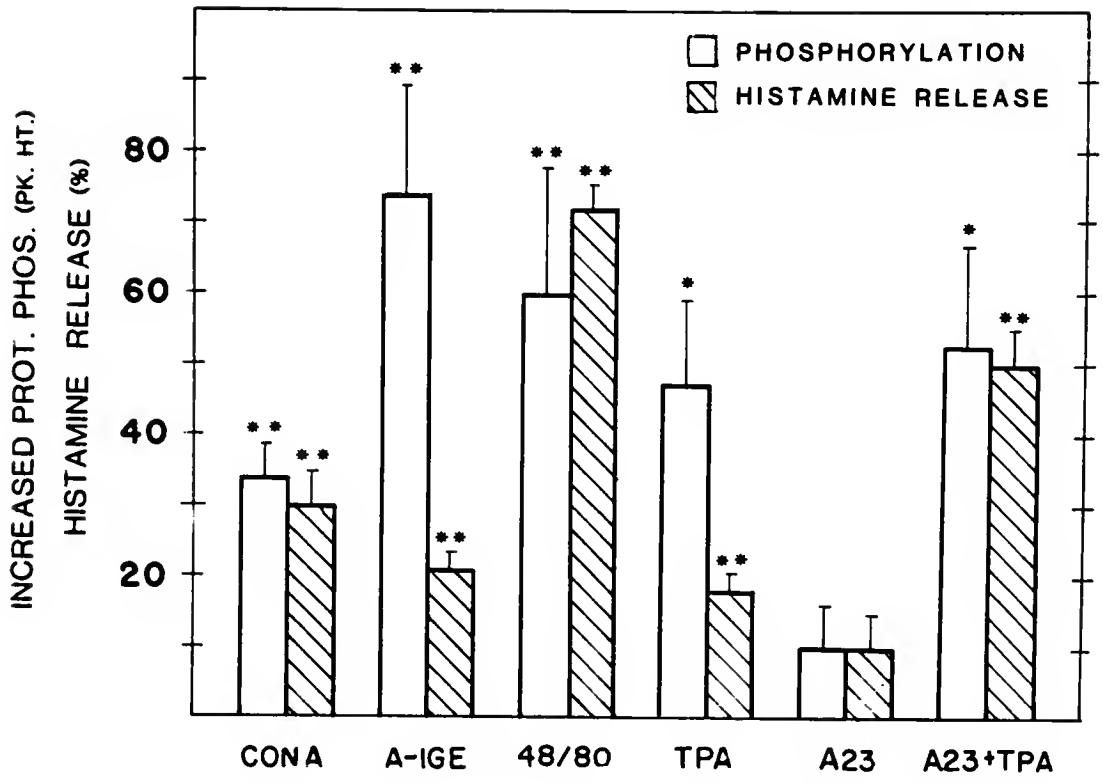

Figure 17. Phosphorylation of a 48 kDa protein during mast cell stimulation. Phosphorylation experiments were incubated 60 sec, histamine release in identically handled, unlabeled cells, for 30 min (also shown in table 3). Subtracted phosphorylation peak heights were: buffer 46 ± 4.4 units; PS 53.2 ± 4.2 units. Subtracted spontaneous histamine release was 6.8 ± 1% for buffer and 9.7 ± 2.3% for PS controls. Secretagogue concentrations are shown in table 3. Values represent the mean ± S.E.M. of arbitrary unit densitometric peak heights of two identically conducted experiments each performed in triplicate. One asterisk indicates those values which differ significantly from controls at $p<0.05$, two asterisks indicate those differing from controls at $p<0.01$, one-way ANOVA.

phosphorylation of the 48 kDa proteins similar to the more classical secretagogues.

The densitometric peak heights of three other major bands (i.e. 78, 59 and 55 kDa) are presented in table 3. Interestingly, phosphorylation of the 78 kDa band was significantly elevated only by compd 48/80. For the 59 kDa proteins, there was a trend toward increased phosphorylation for compd 48/80, con A and anti-IgE with 13%, 12% and 41% increases in peak height when compared with appropriate controls. An exact opposite trend is noted for TPA alone or in conjunction with A23187 where peak heights dropped 17% and 24%, respectively. In the 55 kDa protein band phosphorylations, mean increases in peak heights were: 21%, 48/80; 33%, con A; and 43%, anti-IgE, TPA, A23187 and TPA-A23187 had much smaller effects on the phosphorylation of these proteins. A lack of correlation between phosphorylation and histamine release is not surprising since each secretagogue has a somewhat different time course for release. Phosphorylation changes occurred very fast and were measured after 1 min of stimulation, whereas release was determined 30 min after stimulation. These experiments do indicate that TPA and TPA-A23187 can modify mast cell protein phosphorylation.

Assessment of Mast Cell Ca/PL-PK Activity

Since TPA has been shown to bind and activate the Ca/PL-PK, we compared the activity of this kinase in cerebral cortex, which is known to have a high activity, to that in resident peritoneal cells and purified mast cells. Table 4 depicts results of two experiments showing kinase activity using histone 1 as substrate. By comparison,

TABLE 3

PHOSPHORYLATION OF 78 kDa, 59 kDa, AND 55 kDa PROTEINS DURING MAST CELL STIMULATION

Secretagogue (conc)	Densitometric Peak Heights (Arbitrary Units) Apparent Protein Molecular Weights (kDa) 78	59	55	% Total Histamine Release
Buffer	68.6 ± 5.6	65.3 ± 4.0	90 ± 3.8	6.8 ± 1.0
48/80 (3 μgm/ml)	112 ± 16.4*	73.2 ± 12.6	109 ± 5.8*	79.7 ± 2.7**
TPA (10 ng/ml)	77.4 ± 9.2	52.8 ± 10.2*	97.2 ± 10.4	25.1 ± 2.0**
A23187 (.05 μgm/ml)	64 ± 6.0	120.4 ± 6.4	98.6 ± 8.4	17.4 ± 5.0
TPA + A23187	55.4 ± 7.4	100 ± 12.4*	91 ± 5.0	56.5 ± 4.7**
PS (50 μgm/ml)	73.4 ± 7.6	110 ± 5.4	80.4 ± 9.2	9.7 ± 2.3
con A + PS (10 μgm/ml)	71 ± 7.4	123.6 ± 9.2	106.4 ± 12.0	39.4 ± 4.9**
anti-IgE + PS (1:1000 dil)	76.4 ± 9.4	155.6 ± 15.6*	114.6 ± 3.8*	29.2 ± 2.1**

Experiments were carried as described in Methods and legend of figure 7. One asterisk indicates those values which differed significantly from controls at $p<0.05$ and two asterisks indicate those differing from controls at $p<0.01$, one-way ANOVA. Shown is the mean ± S.E.M. for 6 separate determinations.

TABLE 4

IN VITRO Ca/PL-PK ACTIVITY IN CEREBRAL CORTEX, MAST CELLS AND RESIDENT PERITONEAL CELLS

Tissue	cpm ^{32}P incorporated +PS	cpm ^{32}P incorporated -PS	Ca/PL-PK activity pmol P/μgm protein/4 min
Experiment 1			
Cerebral cortex	74,829 72,143	4753 6027	16.0
RPC - mast cells	34,120 35,434	5516 5329	5.2
Mast cells	5341 4401	2512 2727	0.4
Experiment 2			
Cerebral cortex	34,305 ± 1521	1229 ± 84	8.9
RPC - mast cells	11,488 ± 1102	1240 ± 716	2.8
Mast cells	1542 ± 394	586 ± 3	0.26
RPC	1736 ± 89	948 ± 14	0.21

Enzyme assays on total solubilized fractions were conducted as described in methods with 4 μgm protein per assay tube. Abbreviations used: RPC, resident peritoneal cells were those cells collected by lavage; RPC - mast cells, RPC devoid of mast cells represent a mixed population of white cells which band during metrizamide purification of mast cells. Data presented for experiment 1 are duplicates while those from experiment 2 are mean ± S.E.M. of triplicates.

mast cells had 10-fold and 40-fold less activity than resident peritoneal cells without mast cells (RPC - mast cells) and cortex, respectively. These relationships were not altered when endogenous proteins were used as substrate (data not shown). In RPC fractions which contained approximately 6% mast cells, Ca/PL-PK activity was reduced to the level of purified mast cell homogenate activity. Thus, the presence of mast cells markedly reduced the measured Ca/PL-PK activity in RPC cells suggesting an endogenous inhibitor. Exogenous histamine and heparin did not inhibit Ca/PL-PK activity and other experiments to clearly establish an inhibitor have proved difficult suggesting that any inhibitor that is present is very labile. Since these assays were carried out with optimal conditions for Ca/PL-PK activity, addition of PEs did not significantly increase activity. Unfortunately, the enzyme activity is very low in mast cells making it difficult to demonstrate enhancement of suboptimal activity with either DAG or PEs.

Discussion

We have studied the interaction of PEs with purified rat mast cells. Although PEs alone cause only a small release of histamine, they tremendously potentiate A23187 induced release. Concentrations of ionophore which produce negligible release alone, release approximately 50% of mast cell histamine in the presence of TPA. PEs also synergistically enhance release stimulated by anti-IgE and con A, two secretagogues which release histamine through an interaction with IgE. Polypeptide-base secretagogues such as compd 48/80 and somatostatin, are not potentiated by PEs. In contrast to stimulation involving IgE,

PEs are only additive or less than additive with polypeptide-base secretagogues. Anti-IgE, con A and the calcium ionophore, A23187, all require extracellular calcium to stimulate histamine release, whereas, somatostatin and compd 48/80 do not. Thus, stimulation of histamine release by secretagogues which require extracellular calcium is enhanced by PEs while release by agents which do not require extracellular calcium was not enhanced (Fig. 18).

PEs have been shown to stimulate and potentiate A23187 induced stimulation in a variety of cell types. Studies on human leukocytes have shown that PEs enhance anti-IgE and A23187 stimulated histamine release similar to our findings with purified rat peritoneal mast cells (Schleimer et al., 1982). In addition, the EC_{50} values and overall potency series for the various PEs (e.g., TPA > 4β-PDD > POE >> 4β-PDD and PE) are similar for rat peritoneal mast cells, human basophils (Schleimer et al., 1980), the mouse ear inflammation assay (Driedger and Blumberg, 1979) and platelet serotonin release (Yamanishi et al., 1983). The potency of TPA and the presence of active-inactive isomeric pairs suggests that PEs may be acting at a specific site. The ability of PEs to enhance the response to the calcium ionophore and/or other stimulants known to cause calcium flux suggests that PEs may act by enhancing the sensitivity of cells to calcium activation. Thus, if the concentration of ionophore is proportional to the amount of calcium entering the cell, mast cells are approximately 15-fold more sensitive to calcium (i.e. the EC_{50} for A23187 is reduced 15-fold, see Fig. 13). PEs have been shown to increase the affinity of the Ca/PL-PK for calcium (Kishimoto et al., 1980). Taken together, these findings

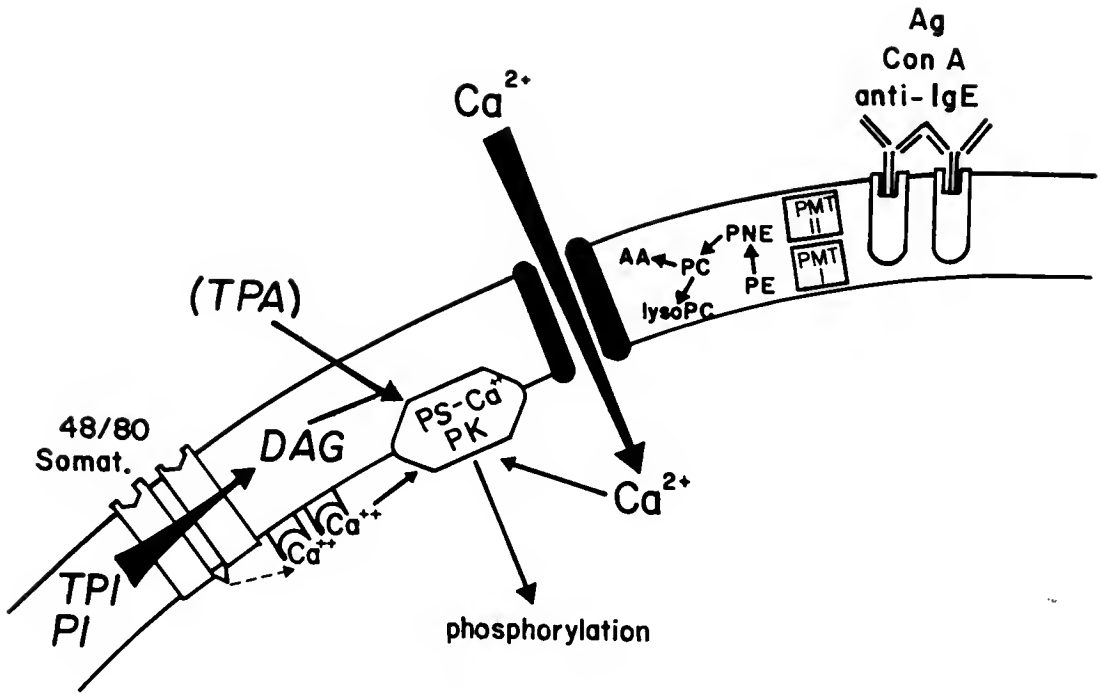

Figure 18. Hypothetical model of the role of Ca/PL-PK in mast cell activation-secretion coupling. Multiple mechanisms may exist for activation of Ca/PL-PK. IgE-like secretagogues may activate the enzyme in the presence of PS primarily through elevation of cytosolic calcium levels by phospholipid methylation. Polypeptide-base secretagogues, which do not significantly alter cytosolic calcium levels, may primarily stimulate the turnovers of phosphatidylinositol forming diacylglycerol which lowers the calcium requirement for the enzyme. Abbreviations used: AA, arachidonic acid; Ag, antigen; con A, concanavalin A; DAG, diacylglycerol; 48/80, compd 48/80; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PNE, phosphatidyl-N-methylethanolamine; PS, phosphatidylserine; somat, somatostatin; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.

suggest that the Ca/PL-PK may be involved in A23187 stimulated histamine release.

PEs bind to a high affinity receptor site in a variety of tissues. Recent studies have found that the PE binding site co-purifies with the Ca/PL-PK. This protein kinase is activated by calcium and phospholipid with phosphatidylserine being the preferred lipid. We have shown that mast cells have Ca/PL-PK activity although not as much as brain tissue or other peritoneal cells. The high activity of rat peritoneal cells with the mast cells removed is likely to be due to neutrophils which are known to have large amounts of Ca/PL-PK (Helfman et al., 1982) and are readily activated by PEs. PEs enhance the sensitivity of Ca/PL-PK to calcium and PS. The PEs apparently mimmic the action of diacyglycerol, a lipid which is formed during mast cell stimulation (Kennerly et al., 1979), and which also enhances the affinity of Ca/PL-PK for calcium and PS. Our finding that exogenous PS and TPA enhance anti-IgE and con A stimulated histamine release in a synergistic manner suggests that the stimulation of secretion by anti-IgE and con A may involve activation of Ca/PL-PK.

Protein kinases and protein phosphorylation-dephosphorylation have been implicated in stimulation secretion coupling in a number of tissues. Previous studies on mast cells have reported changes in the phosphorylation of four protein bands having molecular weights of approximately 42-46, 56-59, 67-68 and 78 kDa during stimulation with compd 48/80 (Sieghart et al., 1978) and anti-IgE (Wells and Mann, 1982). We found that certain secretagogues increased ^{32}P incorporation into proteins having apparent molecular weights of 48, 55, 59 and

78 kDa. Considering differences in requirements for extracellular calcium, PS, and the time course of histamine release, it is not surprising that we found differences in protein phosphorylation 1 min after stimulation. We found our most consistent increases in phosphorylation in the 48 kDa band. Anti-IgE, con A, compd 48/80, TPA alone and TPA-A23187 all markedly increased phosphorylation of this band. PEs and PE-A23187 have been shown to stimulate secretion from neutrophils and platelets, respectively, and to cause prominent increases in the phosphorylation of proteins having apparent molecular weights of approximately 47 kDa and 43 kDa, respectively. Both of these proteins have been shown to be substrates for the Ca/PL-PK. It is possible that these proteins with apparent molecular weights of 42-48 kDa represent closely related proteins which play a role in secretion from a number of cells.

The studies discussed above suggest that the Ca/PL-PK is involved in stimulation-secretion coupling in neutrophils, platelets and other tissues. We have found Ca/PL-PK activity in extracts from rat peritoneal mast cells. Furthermore, our finding that the PEs, which are known to bind to and stimulate the Ca/PL-PK, enhance histamine release by agents which mobilize extracellular calcium and that PEs stimulate protein phosphorylation suggests that activation of the Ca/PL-PK may be involved in histamine release. PEs did not enhance release stimulated by somatostatin or compd 48/80. Compd 48/80 stimulated the largest increases in protein phosphorylation, including the 48 kDa band, and the greatest release of histamine. It is possible that compd 48/80 stimulates secretion through a completely separate

mechanism or that it maximally activates the Ca/PL-PK such that PEs do not further enhance activation. In any case, our results suggest that the Ca/PL-PK may play a role in stimulation-secretion coupling in mast cells.

CHAPTER FOUR
CONCLUSIONS AND SIGNIFICANCE

Experimental results presented in this dissertation demonstrate for the first time that the pharmacological probes hydrocortisone, a prototypic anti-inflammatory steroid, and TPA, a prototypic tumor promoter, exert significant and selective effects upon mast cell stimulated release.

In the case of anti-inflammatory steroid action, we have demonstrated that in cells treated in vitro, long-term (more than 9 hr) pretreatment is required for manifestation of inhibitory effects. Exertion of the inhibitory influences appear to be due to induction of the synthesis of specific proteins. Inhibition of $^{45}Ca^{2+}$ uptake, histamine and [1-^{14}C]-AA release in anti-inflammatory steroid treated cells was limited to secretion by IgE-like secretagogues. Addition of exogenous AA did not overcome the inhibitory effect. No significant inhibition was noted in cells stimulated with polypeptide-base secretagogues or the receptor-independent stimulating agent ionophore A23187.

Other investigators have shown in mouse mast cells, glucocorticoid pretreatment did not alter IgE-Fc receptor number, affinity of IgE for its receptor or basal cAMP levels. Pretreatment did, however, inhibit IgE-induced phospholipid methylation, $^{45}Ca^{2+}$ uptake as well as histamine release. Phospholipase inhibitory protein (lipomodulin) levels were increased in anti-inflammatory steroid pretreated cells.

This evidence suggests that in mast cells, anti-inflammatory steroids exert inhibitory influences early in the biochemical sequence of activation-secretion events. However, several possibilities for glucocorticoid action exist and include: 1) uncoupling cellular activation from influx of extracellular calcium, 2) inhibition of phospholipid methyltransferases, 3) synthesis of phospholipase inhibitory protein which interacts selectively with a subtype of phospholipase associated with Fc receptors, and 4) in a manner analogous to lymphocytes, generation of IgE-suppressive factors, which modulate T cell subset responses to immunologic stimuli may also act selectively in mast cells.

Time courses of glucocorticoid induced inhibition of IgE-like release argue against competition for extracellular calcium binding sites as do the lanthanides, agents which also selectively inhibit IgE-like but not polypeptide-base induced release. Anti-inflammatory steroid induced inhibition is reminiscent of methyltransferase inhibitor action as well as theophylline and dibutyryl cAMP induced inhibition of IgE-antigen elicited methylation, $^{45}Ca^{2+}$ uptake and histamine release. Ionophore A23187 treatment can overcome inhibition by all of these agents, presumably since it functions as a calcium carrier which bypasses receptor-mediated effects which may include both transient cAMP and methyltransferase activation.

As documented earlier, phospholipid methylation has been shown to act prior to calcium influx and glucocorticoids prior to methylation. Thus, it appears that one mechanism of glucocorticoid action in mast cells is by methyltransferase inhibition. This is also the case in

other white cells as Honma et al. (1981) have shown a decrease in phospholipid methylation in dexamethasone treated mouse myeloid leukemia cells during differentiation. In chronic leukemia cells in culture, 6 hr pretreatment with anti-inflammatory steroids did not influence the activity of phospholipid methyltransferase I in forming PME from PE, however, there was a decrease in the conversion of PME to PC by phospholipid methyltransferase II. A similar effect in mast cells could explain glucocorticoid induced inhibition patterns.

Our data does not rule out the generation of phospholipase inhibitory proteins such as lipomodulin as another mechanism of anti-inflammatory steroid action. However, results supportive of a generalized generation of lipomodulin in glucocorticoid pretreated mast cells included only IgE-mediated versus A23187 mediated release. The model is complicated in view of the results of the present investigations where another class of receptor-stimulating agents, polypeptide-base secretagogues were included. Selectivity of the phospholipase inhibitory proteins to IgE-like coupled responses must now be evoked. The fact that exogenously added AA, if it resembles endogenous release, is not sufficient to overcome the presumed phospholipase inhibitory effects of glucocorticoids suggests that other mechanisms may be involved and/or other products of phospholipase activation may be important.

In contrast to the inhibitory effects of glucocorticoids, we have demonstrated that the pharmacologic probe, TPA is capable of increasing mast cell release. In our characterizations of the effects of PEs upon mast cell release, we have shown that, in the presence of extracellular

calcium, PEs tremendously potentiate ionophore A23187 stimulated release of both histamine and AA. PEs act synergistically with IgE-like secretagogues in the presence of PS while interactions with peptide-base types of secretagogues are minimal. Structure-activity relationships for various PE analogues on mast cell histamine release suggest a single mechanism of action, perhaps mediated by a single receptor. In many tissues, the PE receptor is now thought to be a Ca/PL-PK; we have demonstrated low levels of this enzyme in mast cells. Activation of Ca/PL-PK by PEs occurs in the presence of PS by substituting for DAG to increase the affinity of the enzyme for calcium. Our findings that exogenous PS and TPA enhance IgE-like stimulated histamine release in a synergistic manner suggest involvement of the enzyme Ca/PL-PK in mast cell stimulation-secretion coupling. In contrast, peptide-base secretagogues may effect secretion through a completely separate mechanism or through maximum activation of Ca/PL-PK thereby abolishing enhancement by TPA.

In platelets, signal induced PI breakdown is linked to Ca/PL-PK activation by generation of DAG. The synthetic analogue of DAG, 1-oleoyl-2-acetylglycerol (OAG) which gains access to intact platelets with no sign of membrane damage, activated Ca/PL-PK without inducing PI turnover or AA generation. In a similar manner, synergistic effects of OAG and ionophore A23187 have been noted in mast cells. When stimulated with OAG alone, mast cells released histamine to some extent, but in the presence of low concentrations of ionophore A23187, release was dramatically enhanced (Nishizuka, 1984). The suggestion that protein phosphorylation by Ca/PL-PK and the mobilization of

calcium are indispensible and synergistically effective for causing full physiological responses is supported by the following. Turnover of PI and incorporation of radiolabel into PI take place in the mast cell stimulated with antigen, anti-IgE, and con A or compd 48/80 regardless of the presence or omission of extracellular calcium. Thus, PI metabolism may concomitantly participate in activation of Ca/PL-PK, the regulation of calcium channels or mobilization of sequestered calcium, as well as the generation of AA following receptor activation of the mast cell.

McPhail et al. (1984) have recently stated that unsaturated fatty acids, including AA, directly activate Ca/PL-PK in the presence of PS by increasing affinity of the enzyme for calcium. However, at higher concentrations, a loss of enzyme activity which could not be reversed by PS was noted. Like DAG, AA may also have an ability to directly activate and regulate Ca/PL-PK thus giving it a new role in modulation of cellular responses. We have noted, in data not shown, that mast cells can be stimulated to secrete histamine by exogenous AA in the presence of extracellular calcium. That stimulation may be via activation of Ca/PL-PK.

We have also investigated glucocorticoid-TPA interactions and have found that, in the presence of suboptimal concentrations of ionophore A23187, addition of various concentrations of TPA to glucocorticoid pretreated cells did not overcome the inhibition of release of either histamine of [1-^{14}C]-AA. Glucocorticoid pretreatment of mast cells may induce synthesis of a Ca/PL-PK inhibitory protein such as the phorbol ester binding inhibitory factor (PEBIF) described by Hamel et al. (1984).

Relevance of this work toward progress in basic medical research of human allergy is fortified by the similarity of initial triggering events of IgE-mediated release from rat mast cells and purified human lung mast cells.

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BIOGRAPHICAL SKETCH

I, Ann Schwarzbauer, spent my youthful years in Menasha, Wisconsin, involved in the work of dairy farming and in developing an appreciation for plants, animals and space which country life can instill. Adding to this an excellent education at St. Mary Grade and High School, I was given the best of basics with which to begin my adult life.

The following four years of my enrollment in the University of Wisconsin were very eventful and culminated in my Bachelor of Science in medical microbiology. Very shortly thereafter, and perhaps unexpectedly for a farmer's daughter, I married, not a travelling salesman, but Tom, a psychologist.

Our partnership strengthened and developed into personally fulfilling alternations where one of us is employed while the other is a student. My present turn as a student is now closing and with it I have come around full circle from "farmacology" to pharmacology.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 1984

Dean, College of Medicine for 7/12/84

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